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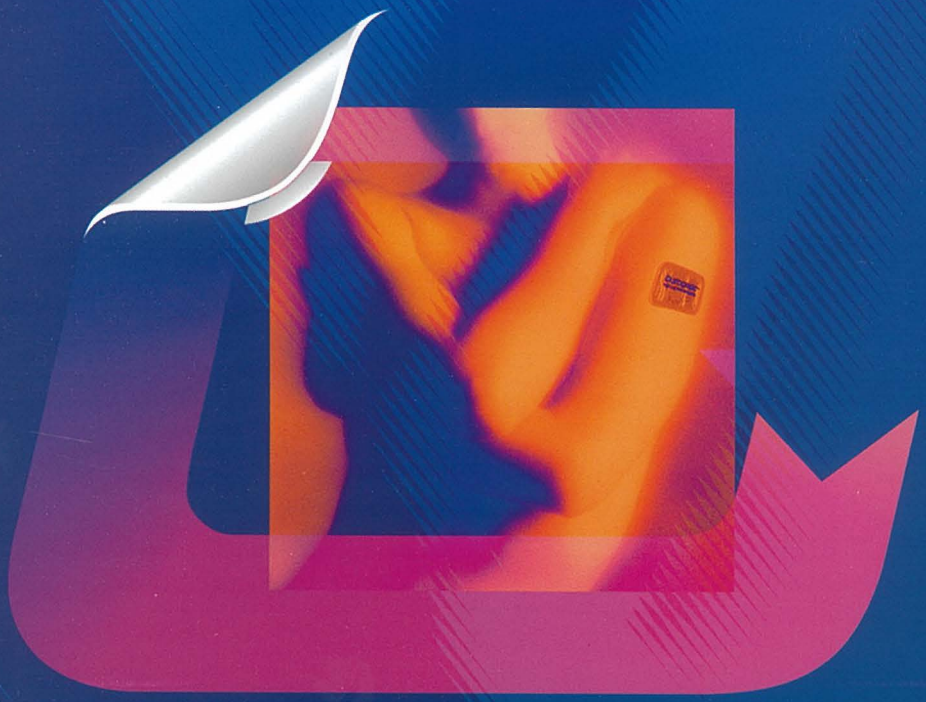

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case report

Incomplete spontaneous ureteral disruption

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Background. *The aim of the authors was to present the case of spontaneous partial ureteral rupture during a renal colic, caused by an anorganic concrement in the proximal part of the left ureter, at the level of the transverse processus of L3.*

Case report. *On the excretory urography imaging, the dilatation of the canal system, cups, necks and pyelon, was observed. At the level of the pyeloureteric passage, the contrast medium was leaking. The leakage was found to be extending along the psoas muscle to the pelvis. On the transversal CT imaging scans, the contrast medium was seen along the medial and dorsal part of the perirenal space, and in the distal part, along the psoas muscle to the pelvis. The ureter was imaged from the pyeloureterical rupture to the site of the concrement. No signs of the damage of the renal parenchyma or perirenal bleeding were detected. During surgery, the site of the rupture was found and also a lot of the perirenal and periureteral liquid. After the extraction of the concrement, the suture of the rupture was made. Postoperative urography and CT showed a normal ureteral image.*

Conclusions. *At the spontaneous partial disruption of the ureter, the contrast medium is still seen in the ureter, distally from the site of the rupture and as extravasation along the psoas muscle.*

Key words: ureter; rupture; tomography, X-ray computed

Introduction

Partial or complete ureteral disruption is often caused by trauma, but can also be a consequence of surgical or other diagnostic procedures. The spontaneous rupture of the ureter is a rare condition, but the most frequent cause is a concrement in the pyelon or ureter. Typical signs for this condition are the absence of renal parenchymal damage, perirenal collection of the urine and absence of haematoma that are always detected in dorsal or medial perirenal space in case of

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trauma.[1-3] After administration of contrast medium, the leakage and extravasation of contrast is always present. In case of the complete disruption, the contrast material is never seen distally from the site of the rupture, in contrast to the partial disruption where the contrast medium is always seen distally.[3-5]

For the differentiation of the disruption type, it is important to make also excretory urography and CT examination with contrast medium in addition to anamnestic or clinical data. We thus have more data on contrast position, haematoma or renal parenchyma damage. The disruption caused by trauma always presents the damage of renal parenchyma and the perirenal haematoma. CT examination is also important in following the position of the contrast and that of the urinoma.[6-8]

Case report

R. N., a 40-year-old woman, was hospitalised because of pain attacks, starting 2 weeks earlier, presenting as a renal colic. The day before hospitalisation, the pain was very strong, with colic characteristic in the area of the left hemiabdomen. Later on, the attack suddenly stopped. Clinical examination detected painful areas at the level of the left kidney and in the left hemiabdomen.

Laboratory examinations were normal, except for a great number of fresh erythrocytes in the urine.

Conventional radiological examination of urotract showed an anorganic concrement with the size of 8 × 4 mm large, at the level of the transversal processus of the L3 on the left (Figure 1).

Excretory urography was made with contrast medium (Telebrix 380, 60 ml). The right kidney was normal, but in the left one, the dilatation of the canal system, including the cups with necks and pyelon, was found. The leakage of the contrast medium from pye-

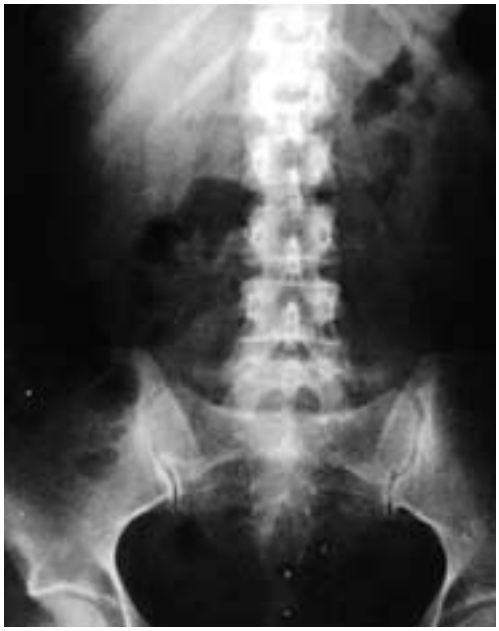

Figure 1. X-ray of abdomen at the level of the transversal processus of L3 on the left, an anorganic concrement, with the size of 8 × 4 mm.

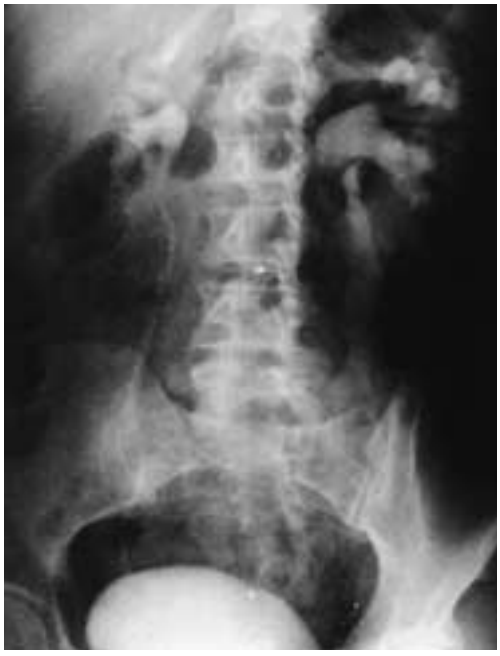

Figure 2. I.v. urography demonstrates the dilatation of the canal system at the left kidney and extravasation of the contrast from the ureter into the periureteral space along the psoas muscle to the level of the pelvis.

loureteric passage in the distal direction along the psoas muscle to the pelvis was also present on the left side. The renal parenchyma on the left was intact (Figure 2).

A conventional CT examination of the abdomen showed the dilatation of the canal system of the left kidney with the extravasation of the contrast medium into the medial and dorsal part of the left perirenal space, and also into the medial and partially ventral part of the peripyelic space. After the introduction of contrast medium, the left ureter shows opacification (Figures 3a,b,c,d). On the distally transverse scans, the contrast medium was seen along the psoas muscle to the pelvis. The findings in the right kidney were normal.

During surgical exploration, a large amount of liquid was found in a perirenal space and along the left ureter and psoas muscle. The site of spontaneous rupture was found at the level of the pyeloureteric junction; the ureter, though, was not completely disconnected from the pyelon. The concrement was extracted through this part of the ureter and surgical suture was made.

All control laboratory examinations were normal. At the control excretory urography performed 5 days after surgical treatment, the dilatation at the middle of the canal system of the kidney was seen, but there was no more signs of the leakage of the contrast.

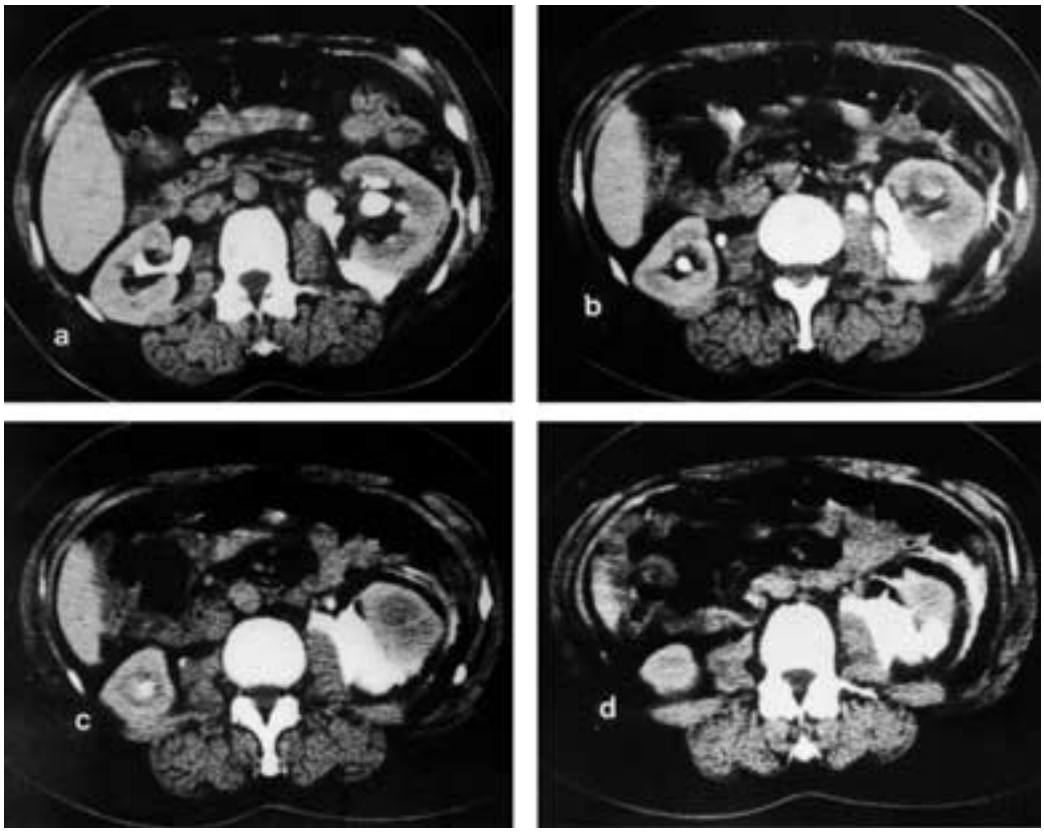

Figure 3. CT finding: **(a)** dilatation of the canal system of the left kidney, with the contrast leaking into the medial and dorsal perirenal space, without any sign of the trauma of the kidney or perirenal haematoma; **(b)** and **(c)** the contrast found in the medial part of the kidney and pyelon; d) the contrast in the ureter dispersed distally along the psoas muscle and also in the periuretral space.

Discussion

Partial spontaneous rupture of the ureter is an extremely rare condition, mostly caused by ureteral stone. The rupture of the ureter and the pyelon with or without the rupture of the kidney can sometime occur as a result of blunt trauma. Such condition can be caused also by surgical or diagnostic ureteral procedures.

The CT image of blunt renal trauma showed the presence of extravasation, laceration and haematoma of renal tissue with perirenal haematoma. Contrary to traumatic disruptions, partial spontaneous and complete disruptions of the ureter have a characteristic CT image after contrast medium applications. This image shows an intact renal parenchyma, the absence of the perirenal haematoma and predominately perirenal fluid collection. At the complete disruption of the ureter caused by trauma the opacifications of the ureter oriented distally from the rupture were not observed, whereas those oriented distally along the psoas muscle is present.

In partial spontaneous disruption of the ureter, the contrast medium in the ureter is seen distally from the site of the rupture and as extravasation along the psoas muscle.

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case report

Gastroparesis in a young diabetic patient

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Background. *Gastroparesis is defined as a delay in emptying of contents from the stomach and occurs in a variety of conditions, e.g. after vagotomy or in systemic diseases such as diabetes mellitus, scleroderma and amyloidosis. The purpose of this paper is to present the radiologic examination, a simple, reliable and noninvasive method as an alternative to other methods for the assessment of gastric emptying.*
Case report. *A twenty-one years old female was admitted because of suspected autonomic neuropathy. She had insulin dependent diabetes mellitus type I for the last ten years. At the time of admission she was complaining of nausea, vomiting, weakness and occasional dizziness. A barium study of oesophagus and stomach was performed: oesophagus was dilated and aperistaltic, remnants of food were seen in aperistaltic stomach but no obstruction, causing delayed emptying, was found.*
Conclusions. *Scintigraphy is at the present the standard technique for the assessment of gastric emptying. Ultrasonography, magnetic resonance imaging, electrogastrography, gastroduodenal manometry and emptying of radiopaque pellets are also used for the evaluation of gastric motility in cases of delayed gastric emptying. The role of barium studies remains to be established.*

Key words: diabetes mellitus; gastroparesis – radiography; contrast media; barium

Introduction

Gastrointestinal motor dysfunction can be the result of many different disorders.[1] In the majority of cases it occurs secondarily to systemic diseases or after abdominal operative procedures,[2] but it is rare as a primary disorder of the autonomic nervous system. Gastroparesis is defined as a delay in emptying of contents from the stomach and occurs after vagotomy,[3] with pancreatic adenocarcinoma, or in diabetes, scleroderma and amyloidosis.[4]

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Diabetes mellitus affects every organ system including the gastrointestinal tract. Almost all diabetic gastrointestinal manifestations are directly or indirectly related to autonomic neuropathy. The basic gastrointestinal abnormality in diabetic autonomic neuropathy is disordered motility.[5]

Abnormal oesophageal motor function is characterised by the absence or decreased amplitude of primary peristaltic wave and by the delay in oesophageal emptying. Lower oesophageal sphincter pressure is decreased and there is a higher incidence of gastroesophageal reflux. Most diabetic patients with disturbed oesophageal motility do not have specific oesophageal symptoms.

Diabetic gastroparesis, associated with delayed gastric emptying of liquids, as well as digestible and indigestible solids, occurs in approximately 25 % of diabetic patients, the majority of them having insulin-dependent diabetes mellitus.[6-8] Many patients with abnormal gastric emptying do not have specific symptoms. The most disturbing are nausea and vomiting, frequently anorexia, early satiety, bloating and discomfort. Gastroparesis contributes to poor diabetic control because of unpredictable oral intake and because of poor absorption as a result of delayed gastric emptying.

The radiologic criteria for gastroparesis are significant solid gastric residue and diminished or ineffectual peristalsis with barium retention at 30 min. without evidence of mechanical gastric outlet obstruction.

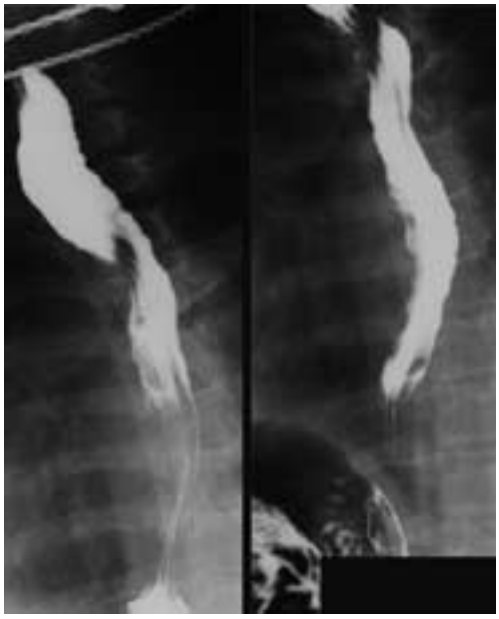

Figure 1. Dilated, aperistaltic oesophagus.

Figure 2. Dilated, aperistaltic stomach, with solid residue, barium contrast in duodenum.

Case report

A twenty-one years old female was admitted because of suspected autonomic neuropathy. She was complaining of occasional vomiting, diarrhoea, general weakness and loss of appetite. She had insulin dependent diabetes mellitus type I for the last ten years, with poorly regulated blood glucose level. Two years previously the findings on endoscopy, performed because of vomiting and epigastric pain, were normal. The patient was referred to ra-

diology for barium examination of the upper gastrointestinal tract because of suspected gastroparesis.

At the examination in the upright position the barium contrast passed freely through the oesophagus, which appeared normal, into the stomach. In prone position the oesophagus was dilated, aperistaltic (Figure 1), the lower oesophageal sphincter was wide open but the contrast did not pass into the stomach. The stomach was dilated, remnants of food were visible, no peristalsis was observed and the contrast passed through the pyloric channel with the patient in the right lateral decubitus position (Figure 2). No obstruction was noted. Normal peristalsis of small bowel was observed. The radiologic diagnosis was gastroparesis with diminished oesophageal motility.

Diagnosis of autonomic neuropathy was confirmed with autonomic nerve tests.

Discussion

Oesophageal motor function can be evaluated by scintigraphy, manometry, 24-hour pH metry, endoscopy and barium swallow,[1,8,9] of which manometry is the most sensitive. Endoscopy and barium swallow are supposed to be structural tests for revealing other causes of slow oesophageal emptying,[7] but radiologic examination of the oesophagus can asses disturbed oesophageal motility, closure of lower oesophageal sphincter and, performed as a double contrast study, can show the changes of the mucosa of the oesophagus.

Gastric emptying function can be evaluated by numerous techniques. On plain abdominal film a dilated stomach with solid residue can be seen in patients with suspected gastroparesis.[5] Barium studies supposedly lack sensitivity, theirs disadvantage being radiation exposure.[7] Scintigraphy is often considered as the gold standard test, but it is expensive and in clinical practice of limited availability, usually restricted to highly specialised medical centres. In a retrospective analysis of gastric emptying of radiopaque pellets the authors concluded that this method is probably a more sensitive test of gastric motor dysfunction than scintigraphy.[10] Ultrasonography requires an expert for interpretation of the study and technically adequate studies are not possible in every subject.[11] Antroduodenal manometry is invasive, not well tolerated in many patients and, as electrogastrography, limited to tertiary centers.[7] Endoscopy is a quite specific structural test, but it is invasive and in the absence of retained food at the time of examination can not rule out gastroparesis.[11] Magnetic resonance imaging is described as an accurate method for evaluation of liquid gastric emptying. It is non-invasive and radiation-free, but it is expensive and not widely available.

In the case of our patient the radiologic examination of the upper gastrointestinal tract with barium revealed absence of oesophageal peristalsis, wide open lower oesophageal sphincter, dilated stomach with remnants of solid food, absence of antral peristalsis and free flow of liquid barium suspension into duodenum, but only in the right lateral decubitus position. Additionally, normal small bowel peristalsis was noted.

In comparison with other tests the radiologic examination is inexpensive, non-invasive and readily available, its potential disadvantages being radiation exposure and, supposedly, low sensitivity.[7] In available literature we could find no record of radiation damage due to the barium studies of the upper gastrointestinal tract. With modern and properly adjusted equipment the radiologic studies can be made virtually "safe",[12] mean doses can be reduced[13] and well within acceptable levels.[14] The examination can be performed as a single contrast study, for the assessment of motility, or/and as a double contrast study, for the assessment of mucosal changes.

Conclusions

The abundance of tests for diagnosis of gastroparesis suggests that each of them has one or even more disadvantages. In our experience the radiologic examination of the upper gastrointestinal tract with barium suspension is more promising in comparison with other tests, being the examination for functional as well as structural abnormalities.

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Magnetic resonance spectroscopy: an overview of the method and its application in clinical neuroradiology

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Background. *Magnetic resonance spectroscopy (MRS) is a comparatively new diagnostic method. Brain tissue is very suitable for MRS analysis. In practice, only a small number of compounds present in the brain may be analysed by MRS. The utility of MRS in neuroradiology and clinical practice is constantly growing, since the investigation is of help in the differential diagnosis of pathological processes as well as in assessing the progress of a disease and evaluating the outcome of treatment. In analysing the data obtained, a number of factors that may influence the objectivity of the result must be taken into account. The magnetic resonance scanner located at the Institute of Radiology, University Medical Centre Ljubljana, utilises modern MRS protocols and has proved a valuable tool in the diagnostic evaluation of neurologic diseases.*
Conclusions. *MRS provides spectral analysis of substance in a selected volume of tissue, thereby offering an insight into the metabolic state of the tissue.*

Key words: nuclear magnetic resonance; nervous system diseases – diagnosis; neuroradiography

Introduction

Magnetic resonance spectroscopy (MRS) is a comparatively new diagnostic method. The aim of this paper is to present briefly this new technique. Specific features of the investigation are outlined, special emphasis being placed on its utility in neuroradiology.

MRS appeared as an analytical method in natural sciences already in 1946[1,2] and has remained indispensable to the present. Magnetic resonance imaging (MRI), permitting visualisation of the examined tissue, was developed only in 1973.[3-5] The method was adopted into clinical practice 10 years later, when computer assisted tomographs with sufficiently powerful and adequately homogeneous magnetic fields became commercially available. Further technical advances in the early 90's made it possible to perform MRS analysis of the patient's tissue in the course of magnetic resonance examination, but these in vivo procedures were at first non-standardized.[6] An important milestone in the evolution of the technique was the development of automated MRS sequences in 1995 [7], which increased the clinical utility of the technique and paved the way for new technological so-

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lutions, resulting in progressive shortening of the investigation and increasing application of MRS in daily radiological practice.

The Institute of Radiology, University Medical Centre Ljubljana, has a modern magnetic resonance tomograph (GE Signa Horizon LX 1.5 T), which allows the use of several modern MRS protocols.

Method

MRS permits in vivo spectral analysis of substance in a selected volume of tissue by means of nuclear magnetic resonance (NMR) and thus offers an insight into the metabolic state of the tissue. In contrast to MRI, which yields information on the distribution of protons in space, displaying the morphology of examined organs, MRS provides chemical analysis of selected tissue.

At present, the majority of clinically applicable MRS protocols employed in neuroradiology make use of the hydrogen nucleus (proton), mainly because of its sensitivity and prevalence in the body. Protons act as small magnetic dipoles in a static magnetic field. If radio-frequency (RF) electromagnetic waves in the form of RF pulses are delivered to tissues in a static homogeneous magnetic field, the tissue receives a certain amount of energy, which may also be absorbed by protons. Protons absorb RF energy only when they are in resonance with a frequency of the delivered RF radiation. This is accompanied by a change in the energy status of the protons, which is a consequence of a different average proton orientation with respect to the direction of the static magnetic field after the RF pulse. On resuming the equilibrium state, the atoms give off the energy in the same form, i.e., as RF radiation, which can be detected by the tomograph with the aid of special RF detection coils. A precondition for high quality signal detection is a strong and highly homogeneous static magnetic field. In MRS the signal is usually acquired from a well-defined volume of the tissue, which is accomplished by the use of special localisation techniques: specially designed sequences of RF pulses [8-12], or with the aid of inhomogeneous RF detection coils (surface coils)[13], or with other less common techniques.[14] Signal detection is followed by computer processing of the acquired data, displayed as spectral analysis of the substance in a selected volume of tissue.

The resonance frequency of protons is proportional to the static magnetic field, i.e., stronger the field higher is the resonance frequency. However, also protons in different chemical groups may have different resonance frequencies. The resonance frequency depends on the type of chemical binding of protons and the influence of surrounding nuclei within individual chemical groups in a molecule. For instance, hydrogen in the group CH_2 has a different resonance frequency from that of hydrogen in the group CH_3, and so these two groups are displayed in the spectrum as two different peaks. The phenomena is called chemical shift, and is expressed with the following equation:

$$\text{Chemical shift (in ppm)} = 1{,}000{,}000\ (\nu/\nu_r - 1) \quad (1)$$

Here νr denotes the resonance frequency of the reference standard (plain water) and ppm stands for parts per million.

The spectrum is represented as a graph in which the values of the difference in the resonance frequency of chemical groups from the reference value, indicating the type of chemical group e.g. the type of substance, are presented on the *x*-axis. On the y-axis, the surface under individual peaks of the curve reflects the concentration of a specific chemical group and is therefore proportional to the amount of individual substances in the selected volume. The units on both axes are relative. Values on the x-axis are expressed in ppm (parts per million) and on the ordinate in re-

lative concentration (percent). The spectra obtained are the result of a large number of repeated measurements and represent averaging of the measured values. With MRS it is possible to analyse chemical groups with unbound protons. For their detection, the concentration of the substance in the selected volume of tissue must not be less than 0.5-1 mM/kg of tissue.

In addition to the spectrum, the computer calculates and displays also the ratios between selected substances. In standardised protocols, the types of substances to be compared are determined in advance. These ratios are very important in interpreting the results since they allow the elimination of various factors that affect the results of measurements (osmotic status of the tissue, presence of paramagnetic and ferromagnetic substances etc.).

In order to obtain a technically adequate spectrum, we must conduct the investigation in specific phases. Calibration of the instrument involves adjusting the gain of the transmitter to optimise RF pulses in the sequence. Also the gain of the receiver needs to be adjusted to allow the optimal digitalisation of the received analogue signal. Since every sample spoils homogeneity of the static magnetic field due to its magnetic susceptibility a correction of the static magnetic field homogeneity is needed. This procedure is called shimming and is done by the use of various coils in the magnet, which may produce additional inhomogeneous static magnetic fields of adjustable amplitudes to the main static magnetic field. Suitable combination of these fields may improve the static magnetic field homogeneity. Shimming is essential for MRS. Shim adjustment is followed by adjustment of the signal suppression pulses. Namely, predominant substance in the tissue, such as lipid or so-called free water, would have in the spectrum extremely high spectral line that would overwhelm all other spectral lines of much higher interest. Suppression of the predominant substance is therefore necessary. By graphic localisation of the volume of interest in the MR image, we define the site and size of the volume of tissue to be analysed. Sequence parameters set-up is followed by the execution of the pulse sequence in two phases. During the first phase the magnetic resonance signal is excited in the selected volume of the tissue by RF pulses of a special shape, amplitude and duration. This is followed be the second phase during which magnetic resonance signal is acquired. The measurements are repeated many times and the results are averaged. Finally, a spectrum is calculated from the acquired time domain data by the mathematical transformation known as the Fourier transformation. Spectrum post processing is also possible as for example noise filtering, phase correction and line-width measurements. In modern MRS protocols, all important phases are automated, whereby the duration of analysis is appreciably reduced.

The results of investigation are affected by a number of factors. Important technical factors include strength of the basic static magnetic field, size of the selected volume of tissue, number of measurement repetitions, selected technical parameters (echo time, repetition rate) etc. The homogeneity of the local magnetic field in the selected volume of tissue is affected by tissue structure, presence of paramagnetic substances (e.g. contrast medium) and ferromagnetic substances (e.g. blood), and concentration of osmotically active substances and electrolytes (e.g. mannitol).

Several types of MRS are known. They can be classified according to observed nuclei, which are most often hydrogen, but carbon, fluorine, phosphorus or some other nuclei can also be observed. The most widely used methods for spatial localisation of the sample are volume methods, such as the STEAM (stimulated echo acquisition mode)[13] and the PRESS (point resolved spectroscopy)[12] spin echo sequences, which utilise selective RF pulses to

define the volume of interest in the sample and compare it to slices in the MR image. At present, PRESS is the preferred method, mainly because of a better signal-to-noise ratio. Tissue can be simultaneously analysed in a single volume of interest (single voxel spectroscopy, SVS) or in several volumes of interest (multi voxel spectroscopy, MVS) in selected planes (2D or 3D). If the results of analysis are superimposed on the morphological image of an organ, we speak of so-called spectroscopic imaging. In this mode of MRS, the computer assigns each metabolite an appropriate, so that the distribution and intensity of colours in the image of an organ illustrate the distribution and concentration of individual metabolites in the tissue being analysed.

In our work, we now use routinely an automated pulse sequence, PRESS-SV (General Electrics) with the following parameters: repetition time TR = 1500 ms, echo time TE = 35/144/288 ms, voxel size 2 × 2 × 2 cm, NEX 8, FOV 24.

Preparation of the patient for the investigation, positioning in the MR tomograph, safety precautions and other measures are the same as for MRI. During the procedure, the patient, or the tissue being examined, must be completely motionless. Therefore restless patients and children are examined in general anaesthesia. Individual spectroscopic analysis at a single volume of interest lasts about 10 minutes. As 6-9 analyses are usually required, the examination may take as long as 90 minutes. Therefore MRS is regarded as a special radiological procedure, which should be undertaken only on the basis of proper indications. Lesions located on the border between tissues of different composition (e.g. brain / skull, brain / liquor space) or close to a bleeding site are difficult to analyse, as the local magnetic field in such regions is very inhomogeneous. Similar problems occur with processes involving tissues with a high content of water or lipids, where suppression of the signal during data processing is difficult and often inadequate. It is important to select a solid segment of pathological tissue that adequately reflects the primary pathological process (e.g. the edge of a lesion) and not an area showing secondary changes (necrosis etc.). Selection of the volume of tissue to be analysed, or its location, is thus of vital importance for the technical quality of the spectrum as well as for proper interpretation of the result.

Clinical value

MRS can be used for the analysis of all pathological processes in tissues fulfilling the previously mentioned conditions (muscles, parenchymal organs etc.).

MRS in neuroradiology[15,16]

In further text, we will concentrate on the use of MRS in neuroradiology, where the technique has found the widest application. Brain tissue is stationary, mostly homogeneous and therefore suitable for MRS. Moreover, it is not readily accessible for invasive cyto-histological investigations, which carry a substantial risk of procedure-related complications. The main indications for MRS are inborn and acquired metabolic disorders, neonatal hypoxia and other sequelae of ischaemic brain injury, disorders of myelination, degenerative brain diseases, epilepsy, infections, brain tumours and others. MRS provides an insight into the metabolic state of the selected tissue and assists both in the differential diagnosis of pathological processes and in assessing the progress of illness (e.g. tumour malignancy) and outcome of treatment (e.g. evaluation of residual tumour). In practice, only a small number of neurochemical compounds present in brain tissue can be analysed by MRS. The main metabolites (shown with the abbreviations and main resonance frequencies) are: N-acetylaspartate (NAA: 2.0 ppm), choline and choline-rich compounds (Cho: 3.22

ppm), creatine (Cr: 3.0 ppm), lactate (Lac: 1.3 ppm), lipids (Lip 0.9 and 1.3 ppm). In certain pathological processes, other metabolites, which may be typical or non-specific, occur at their resonance frequencies.

NAA is found in neurones and is an indicator of the neurone / axon density in brain tissue. Its precise role has not been definitely established.

Creatine originates mainly from the intracellular pool of creatine and phosphocreatine and is an indicator of the energy state and activity of cells.

Most of *choline* present in brain tissue is under normal circumstances inaccessible to MRS analysis as it is bound to cell membranes, myelin and other complex lipoproteins and/or lipids. Its two main constituents of choline pool, phosphorylcholine and glycerophosphorylcholine, making the largest contribution to the measured choline value (choline peak in the spectrum), are released in increased quantities during degradation and/or increased turnover of cell membranes and myelin sheaths.

What has been said about choline applies also to free lipids, whose concentration is related to the previously mentioned pathological processes, and which are frequently found in association with altered choline metabolism.

Lactate is not detected by MRS in normal brain tissue. However, its concentration is increased in anaerobic metabolism or accelerated glyosis.

In some pathological processes, increased concentrations of some additional, non-standard compounds may be registrated. So the concentration of certain amino acids is elevated in infected tissue as a result of metabolism in the presence of bacteria (abscesses), and precursors are elevated as a result of impaired metabolism of normal substances (enzyme defects) etc.

Analysis of the results of investigation requires caution and experience. The spectral peaks of metabolites may overlap because of equal resonance frequencies. The concentration of compounds varies in different parts of the brain (grey matter, white matter, basal ganglia), and so samples from different regions should be analysed. The content of substances depends also on the subject's age. The concentration of metabolites is affected by the presence of osmotically active substances (e.g. mannitol) and drugs in the brain. Ferromagnetic and paramagnetic substances, such as contrast agents (gadolinium, haemoglobin degradation products) affect the MR properties of metabolites. In patients whose brain is not diffusely involved, these problems may be overcome by comparison of spectra from the healthy and affected sides.

At the Institute of Radiology, University Medical Centre Ljubljana, MRS is currently used routinely only in neuroradiology, mainly for neuropaediatric diagnosis and for the diagnostic workup and evaluation of the results of treatment of brain tumours. However, the indications for the investigation are constantly widening.

In the *future*, major advances may be expected to take place in MRS as a result of progressively faster protocols, better signal-to-noise ratio and new combinations of different techniques and types of spectroscopy, exploiting different kinds of reference elements.

Case report

In a 29-year-old male patient with frequent absence seizures, CT and MRI revealed a space-occupying lesion of undefined aetiology in the left temporal region, which was compatible with vasculitis or a low-grade neoplasm (Figure 2). The patient underwent an operation, comprising uncotomy, hypocampotomy and removal of the medial part of the left temporal lobe. Histological examination of the removed tissue failed to rule out either of the two possible diagnoses. After the operation,

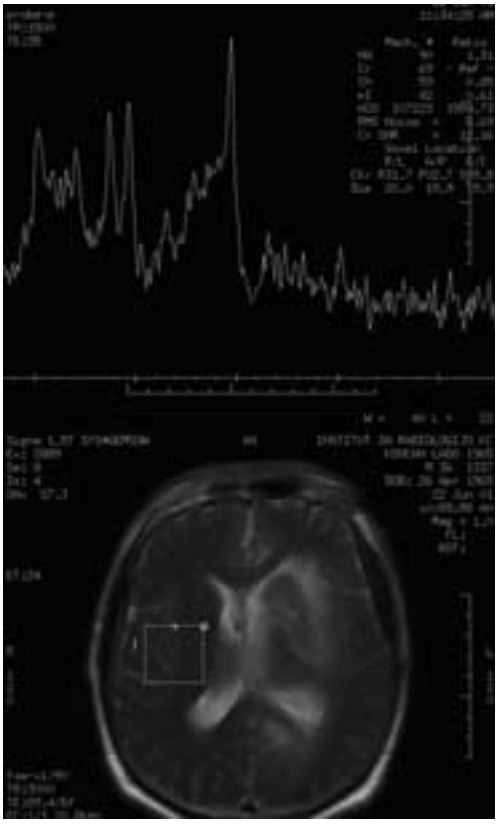

Figure 1. Normal, technically appropriate spectrum (above) from the designated area of the brain (below). Values on the *x*-axis reflect the type of compound, and those on the *y*-axis the concentration of this compound. The ratios between selected substances and the technical parameters of the investigation are presented.

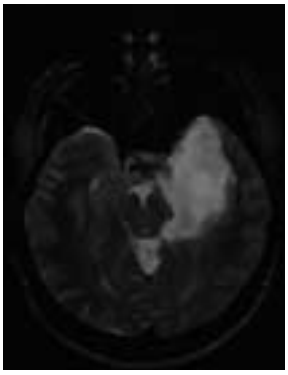

Figure 2. T2-weighted MRI obtained prior to surgery, showing oedema of brain tissue in the left temporal region.

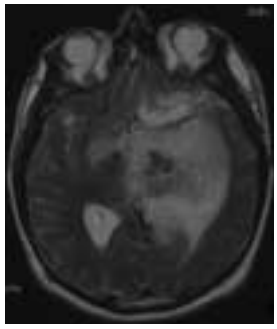

Figure 3. T2-weighted MRI obtained after surgery, showing a postoperative defect in the left temporal region. There is persistent oedema within the hemisphere, encroaching upon surrounding structures and causing progressive hydrocephalus.

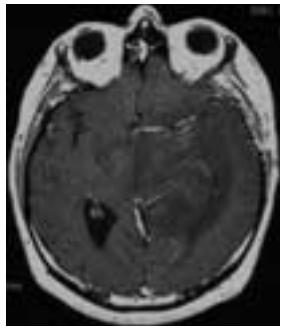

Figure 4. T1-weighted MRI obtained after application of contrast medium, showing the state after operation. The lesion does not enhance after gadolinium application.

the seizures persisted, and the patient continued to receive antiepileptic therapy and corticosteroids. Follow-up CT a year after the operation showed persistent oedema in the operated area. Consequently MRI and MRS were repeated as well. MRI confirmed the presence of a space-occupying, partly infiltrative lesion with surrounding oedema in the left temporal region, which extended into the depth of the hemisphere, encroaching upon adjacent structures and causing increasing hydrocephalus (Figure 3). It did not enhance after gadolinium application, which spoke in favour of a low-grade tumour (Figure 4). MRS was pathological, showing a reduced concentration of NAA and significantly increased

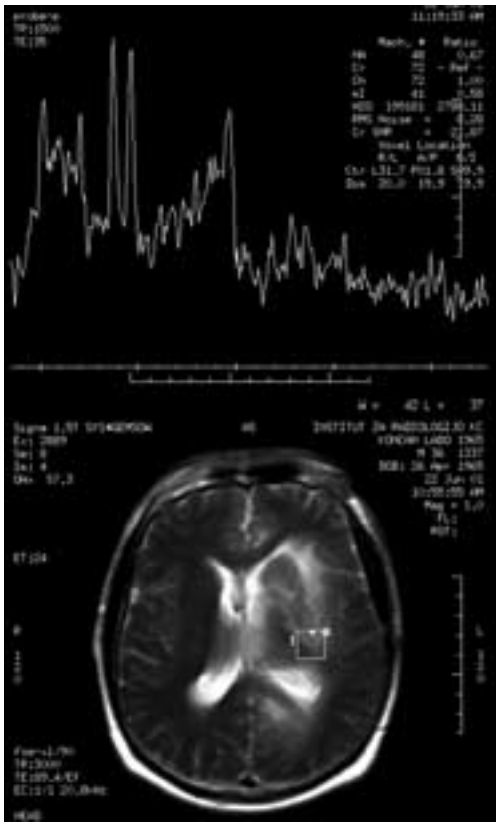

Figure 5. Magnetic resonance spectroscopy (above) in a selected volume of pathological tissue (below). The concentration of NAA is reduced, while the concentrations of choline and lactate are significantly elevated. The spectrum is typical of a low-grade malignant process.

concentrations of choline and lactate, compatible with a low-grade tumour (Figure 5). A reoperation was performed, and histological examination of the removed tissue yielded conclusive evidence of a low-grade tumour (glioma).

Conclusions

MRS provides spectral analysis of substance in a selected volume of tissue, giving information on the metabolic state of the tissue. Brain tissue is very suitable for MRS analysis. In practice, only a small number of compounds present in the brain may be analysed by MRS. The utility of MRS in neuroradiology and clinical practice is constantly growing, since the investigation is of help in the differential diagnosis of pathological processes as well as in assessing the progress of a disease and evaluating the outcome of treatment. In analysing the data obtained, a number of factors that may influence the objectivity of the result must be taken into account. The magnetic resonance scanner located at the Institute of Radiology, University Medical Centre Ljubljana, utilises modern MRS protocols and has proved a valuable tool in the diagnostic evaluation of neurologic diseases.

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Without my friend Dr. Igor Serša, who helped me with references, comments and suggestions regarding physics of MRS, I would probably never dare to finish this paper.

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review

Advances in contrast-enhanced MR-angiography: Indications and limitations

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Introduction. *Gadolinium(Gd)-enhanced three-dimensional (3D) magnetic resonance angiography (MRA) is a newer technique that provides high resolution data rapidly for depiction of both arteries and veins throughout the body.*

Conclusions. *Contrast-enhanced 3D MRA represents a milestone for non-invasive vascular imaging. While its clinical utility has already been established in many vascular territories, the continuos development of hard- and software, as well as of new contrast agents, will likely result in a further widening in the spectrum of indications. Delayed 3D acquisitions of the kidney, ureters and bladder can be performed routinely to demonstrate and characterise obstruction, delayed function, filling defects and masses. Some patients are not candidates for MRA because of pacemakers, some kind of stents or immobilisation coils that cause considerable artifacts and obscure important structures. The resolution of CE-3D-MRA is lower compared with that of conventional angiography and visualisation of small peripheral arteries is limited.*

Key words: magnetic resonance angiography – methods; contrast media

Introduction

Gadolinium(Gd)-enhanced three-dimensional (3D) magnetic resonance angiography (MRA) is a newer technique that provides high resolution data rapidly for depiction of both arteries and veins throughout the body.[1]

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Improvements in gradient technology now allow an entire volume of 3D-Gd-enhanced MRA to be performed at 4 second intervals. It is now possible to acquire several volumes of high temporal resolution 3D data sets in a 10-20 second breath hold. Because it relies on T1 shortening effects of circulating Gd-chelate contrast media and not inherent flow characteristics, Gd-enhanced 3D-MRA can often depict pathologic vascular segments that are not adequately visualised using unenhanced flow based MRI-techniques. In addition, Gd-enhanced 3D-MRA provides volumetric data that can be processed for multiplanar refor-

mation (MPR) and maximum intensity projection (MIP) viewing.

This article will review the basic techniques for MR-angiography with contrast media and the potential applications of MRA will be discussed with particular emphasis on the 3D-contrast-enhanced technique.

Clinical applications

Paramagnetic contrast agents shorten the T1 relaxation time of blood. Gadolinium has a high relaxivity and a favourable safety profile when bound to a chelate.[2] During the short intravascular phase the intravenously injected T1-shortening contrast agent provides a signal in the arterial or venous system, thereby enhancing the vessel to background contrast to noise ratio and eliminating flow artifacts. Hence signal of flowing blood is no longer flow-dependent. Flow-induced artifacts seen with time of flight (TOF) or phase contrast MRA are therefore largely eliminated. This allows coverage of large vascular territories in short imaging times and generates images, which are similar in appearance to conventional contrast angiography.

Beyond the absence of ionising radiation, side effects of the paramagnetic contrast agents used for the examination are rare. Paramagnetic contrast agents are non-nephrotoxic and have a low incidence of allergic reactions. They are safe for use in patients with renal insufficiency as well as in patients with a history of allergic reactions to iodinated contrast media.

To achieve maximal T1-weighting, the sequence employed should be spoiled.[3] Repetition and echotimes should be chosen as short as possible; a flip angle ranging between 20° and 45° provides adequate suppression of the surrounding tissues and has been shown to render excellent image quality.

Section thickness should be adjusted to between 1.5 and 2.5 mm in order to assure for full coverage of the vascular system under consideration and still permit multiplanar reformations.

Breath-holding is crucial for achieving a good 3D-MRA image quality in the thorax and abdomen. The breath-hold interval can be increased by allowing the patient to practise before the exam and by providing some oxygen through a nasal canula.

It is essential to inject sufficient paramagnetic contrast agent to reduce arterial blood T1 to well under the T1 of surrounding tissues. Even though most extracellular agents available in European markets are approved up to a dose of 0.3 mmol per kg, cost considerations require careful dosing independent of the vascular territory under consideration. A dose ranging between 0.1 and 0.2 mmol per kg body weight is sufficient for most single-territory artery imaging protocols. Display of the run of arteries in multiple stations requires administration of the maximum allowable dose of 0.3 mmol per kg body-weight.[4]

If data collection is sufficiently fast for time resolved imaging, optimal image quality is virtually guaranteed in at least one of the collected data sets. Otherwise, timing of the contrast bolus is crucial (with a test bolus injection or automated bolus detection). The use of an automated injector facilitates contrast timing and delivery as it allows precise infusion of paramagnetic contrast using predefined weight adjusted rates and volumes. Maximal contrast concentration in the vessel of interest should be achieved during the acquisition of the central, contrast-determining portion of k-space.[3]

Image analysis

Analysis should not be limited to viewing maximum intensity projections but instead should include evaluation of reformatted images in all three planes and should include also the evaluation of the source images, viewed interactively on a work station.

1. Pulmonary arteries: Using a breath hold technique, the pulmonary artery tree can be visualized to its periphery (Figure 1). Contrast-enhanced 3D-MRA has also been shown to be a save and reliable technique for the detection of pulmonary embolism. Emboli are clearly identifiable on the reformatted images as filling defects.[5]
2. Aorta: Contrast-enhanced 3D-MRA is emerging as the imaging modality of choice for assessing the whole aorta as an organ. Employing the described strategy all relevant disease processes of the aorta as well as the major neck and arm vessels can be fully depicted on the 3D-MR-angiography images. The possible complex morphology of the diseased aorta and/or its branches may result in an inadvertent exclusion of important portions of the arterial anatomy from the 3D imaging volume. Therefore it is important to carefully conduct the localising process using breath-hold techniques. Stenoses are visualized as well as aneurysmal dilatations. Congenital lesions, such as coarctations, are particularly well suited for analysis with this technique. In addition, surgical grafts are well depicted. Furthermore MRA provides comprehensive analysis in suspected aortic dissection: The extent and relationship to branch vessels can be fully depicted and the true lumen can be separated from the false lumen (Figure 1).[6]

For evaluation of the aorta the advantages of cross sectional imaging over conventional catheter angiography is well established.

Furthermore it is possible to evaluate the renal arteries to the level of the renal hilum, as well as the superior mesenteric artery and even the celiac trunc and the inferior mesenteric artery.

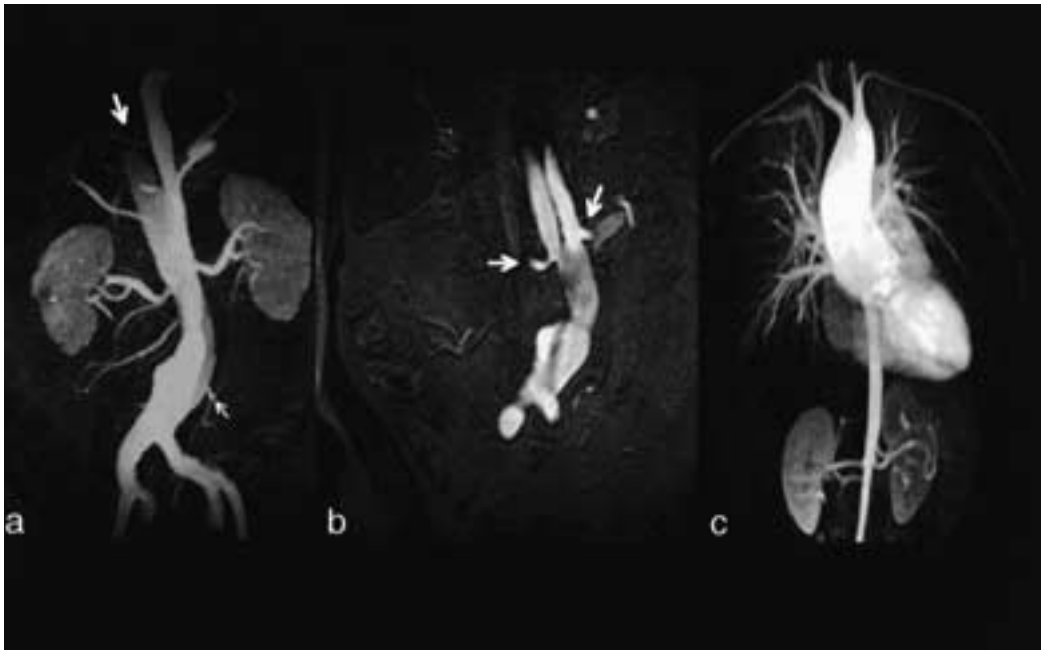

Figure 1. MR angiography of the aorta showing
1. dissection (a,b) with exact delineation of true and false lumen and the dissection membrane, notice inferior mesenteric artery.
2. ascendens aneurysma in Marfan's Syndrom (notice also pulmonary arteries).

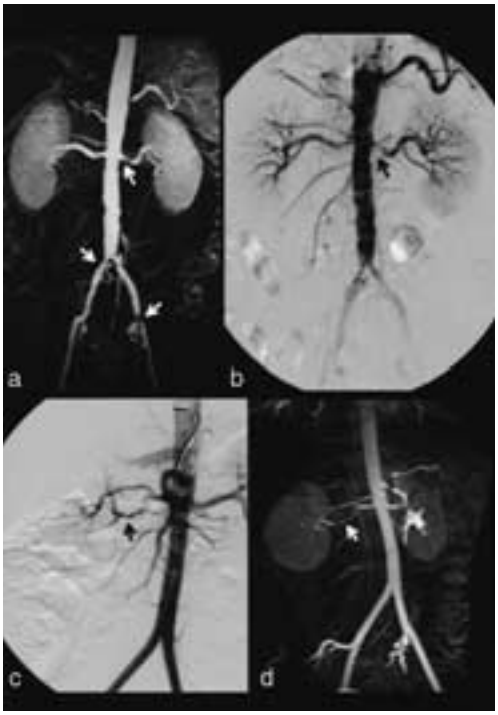

Figure 2. Renal angiograms showing
1. exact correlation between a) MRA and b) conventional angiography regarding the high grade renal artery stenosis with better delineation of pelvic artery stenoses in MRA.
2. discrete renal artery irregularities in fibromuscular dysplasia in d) MRA and c) conventional angiography.

With sensitivities and specificities up to 100 % it appears likely that the 3D-MRA techniques will replace conventional angiography (Figure 2) as a primary means for the assessment of renal artery disease.[7] 3D-MRA is also the modality of choice for assessing complex arterial anatomy following renal transplantation.

3. Peripheral vessels: Also in the peripheral vasculature the contrast-enhanced approach offers several advantages over conventional MRA-techniques including rapid acquisition without arterial puncture. This technique uses several 3D-MRA-acquisistions in conjunction with movement of the MRA-scanning table to follow the bolus of contrast into the lower extremities (Figure 3).[8,9]

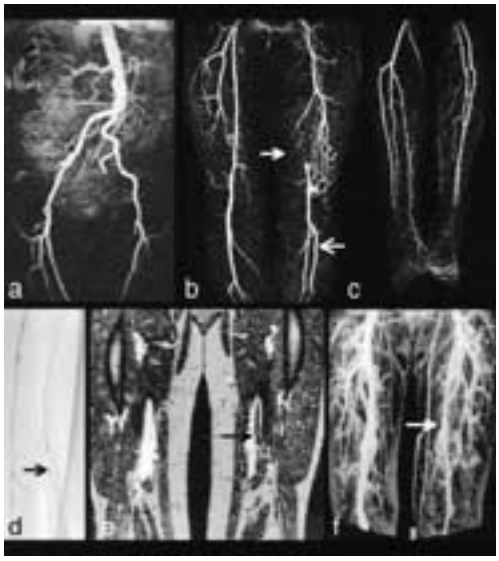

Figure 3. MR angiography of the outflow-tract:
1. with Gadolinium (a,b,c): chronic long occlusion of the left femoral superficial artery and double popliteal artery
2. with blood pool agent (e,f): fresh thrombus visible in the superficial femoral vein according to conventional phlebography (d).

Three or even more 3D-data-sets, each consisting of 30 to 40 images are collected at set intervals over the lower extremities, resulting and a morphologic display of the entire run off system. To avoid venous overlap, which might mask patent arterial segments, imaging has to be completed before a too much contrast is present in the venous system. Therefore, for moving bed imaging a low infusion rate for the administration of contrast agent (0.3 to 0.6 ml per sec) is recommended to minimize venous enhancement. So the clinically relevant vascular pathologies are well displayed as confirmed in recent studies. Contrast-enhanced 3D-MRA achieves sensitivity and specificity values exceeding 90 % in differentiating non-significant from haemodynamically significant stenosis.

4. Whole body 3D-MRA: Since arteriosclerotic disease effects entire arterial system, extended coverage allowing the concomi-

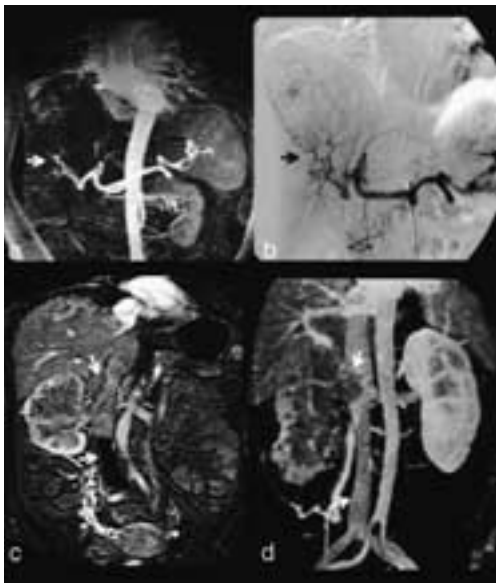

Figure 4. Abdominal angiograms in oncologic patients:
1. showing preembolization feeding vessels in a) MRA and b) conventional angiography (hepatocellular carcinoma).
2. differentiation between c) tumour thrombus (proximal) and clot (distal) in the VCI (renal cell carcinoma) and d) partial tumor ingrown with patent VCI (also renal cell carcinoma).

tant assessment of the arterial system from supraaortic arteries to the distal run of vessels appears desirable. Five 3D data sets are collected over 72 seconds during a continuous contrast infusion lasting 60 seconds. It allows displaying of the arterial vascular tree from supraaortic arteries to distal run of vessels in a single examination lasting nearly 72 seconds.[10]
Extended coverage including the entire arterial vascular tree, high diagnostic accuracy, non-invasiveness, lack of side effects and very short examination times combine to open interesting perspectives regarding the use of this technique for vascular disease screening.

5. Systemic veins
Evaluation of the systemic veins not only of the abdomen with 3D-MRA is usually optimal on the second or third postcontrast acquisition. Applications include evaluation of patients with suspected venous thrombosis, vascular extension by renal, hepatic or other tumors and evaluation of anatomic anomalies of the inferior vena cava and systemic veins (Figure 4).[11]

Future developments

Blood pool or intravascular contrast agents are large enough that they do not leak out of the capillaries but stay within the intravascular compartment for more than 1 hour.[2] At this time it appears likely however, that these agents will enhance imaging of the venous system as well as of the coronary arteries (Figures 3 e,f).

Conclusions

Contrast-enhanced 3D MRA represents a milestone for non-invasive vascular imaging. While its clinical utility has already been established in many vascular territories, the continuos development of hard- and software, as well as of new contrast agents, will likely result in a further widening in the spectrum of indications. Including MR-imaging MRA allows the one-stop shop examination of the most pathologies of the abdomen, neck and extremities as well as the thorax. Delayed 3D acquisitions of the kidney, ureters and bladder can be performed routinely to demonstrate and characterise obstruction, delayed function, filling defects and masses. CE-3D-MRA is not without limitations. Some patients are not candidates for MRA because of pacemakers, some kind of stents or immobilisation coils that cause considerable artifacts and obscure important structures. The resolution of CE-3D-MRA is lower compared with that of conventional angiography and visualization of small peripheral arteries is limited.

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review

Radiotherapy- and chemotherapy-induced normal tissue damage: the role of cytokines and adhesion molecules

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Background. *Ionising radiation and cytostatic agents used in cancer therapy exert damaging effects on normal tissues and induce a complex response at the cellular and molecular levels. Cytokines and adhesion molecules are involved in this response.*
Methods. *Published data on the given topic have been reviewed.*
Results and conclusions. *Various cytokines and adhesion molecules, including tumor necrosis factor α, interleukins-1,-2,-4, and -6, interferon γ, granulocyte macrophage- and macrophage- colony stimulating factors, transforming growth factor β, platelet-derived growth factor, insulin-like growth factor I, fibroblast and epidermal growth factors, platelet-activating factor, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E- and P-selectins are involved in the response of normal tissues to ionizing radiation- and chemotherapy-induced normal tissues damage and are co-responsible for some side effects of these treatment modalities, including fever, anorexia and fatigue, suppression of hematopoiesis, both acute and late local tissue response.*

Key words: cytokines – radiation effects – drug effects; antineoplatic agents – adverse effects; neoplasms – drug therapy – radiotherapy; cell adhesion molecules – radiation effects – drug effects

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Introduction

Injury of human tissues and generally of mammalian organism ones, activates a non-specific, but highly complex immune response at the intracellular and intercellular levels, with the aim to protect the tissues and the whole organism against exogenous damage and to regenerate the damaged tissues. Cytokines and adhesion molecules are released during this response and mediate intercellular interactions among effectors of immune and other systems.[1-3]

Cytokines are soluble polypeptides regulating and determining the character of immune response.[1,2] The main source of cytokines are macrophages, but neutrophils, lymphocytes, platelets, endothelial cells, fibroblasts, and microglia, acting as the macrophage of the central nervous system (CNS), are able to release cytokines as well.[3-5] Cytokines are components of a large, complex signalling network. The great variety of cell types that are able to release cytokines and the great diversity of biological effects of each cytokine is confusing. The ability of individual cytokines to induce or inhibit the synthesis of other cytokines and often of its own further complicates the specification of biological functions of individual cytokines.[1, 2, 6]

Adhesion molecules mediate the adherence of leukocytes to the molecules on other cells or to extracellular matrix ligands and are thus involved in leukocyte activation, circulation and localization to inflammatory sites.[7]

Both radiotherapy and chemotherapy exert damaging effects on normal tissues in cancer patients and, consequently, induce an immune response in these tissues. The role of cytokines in this response and the possibilities to modulate it in order to lower the risk of side effects of these treatment modalities are reviewed in this article.

Ionizing radiation- and chemotherapy-induced cytokines and adhesion molecules

The production of cytokines may result from either DNA damage in cells leading to inhibition in cell cycle progression and resulting in cell death [8,9] or from biochemical changes in cellular environment and metabolism induced by the interaction of ionizing radiation (or chemotherapy) with the target cell (Figure 1).[3]

The cytokines and adhesion molecules that have been observed to be produced in response to ionizing radiation at the mRNA or protein levels in various human or other mammalian cells or tissues both in vitro and vivo are summarized in Table 1. The mediators have been shown to respond to irradiation in a dose-dependent manner.[22, 25, 31, 40, 44, 51, 52] The threshold dose of irradiation ranges from 0.5 to 2 Gy for different proteins, except murine brain cells where the threshold dose of 7 Gy has been found.[21] Their production is also time-dependent, peaking usually at 4-24 hours after irradiation with subsequent decrease to normal levels within 24 hours to a few days.[21] Increased intercellular adhesion molecule-1 (ICAM-1) expression persists for at least several days; [46, 47, 51] expression of transforming growth factor-β (TGF-β) is often delayed to weeks or months after irradiation and persists for months; [38, 40] in the murine lung and small intestine, increased levels of interleukin-1 (IL-1), IL-4, tumor necrosis factor-α (TNF-α), and platelet derived growth factor (PDGF) also persist for weeks and months after irradiation; [18, 29, 34] reelevation of TNF-α at 2-3 months and its continued overexpression more than half a year after irradiation has been observed in brain cells.[21]

Although the immune response to chemotherapeutic drugs has not been studied as extensively as that to irradiation, it is highly probable that the administration of such toxic and aggressive agents as anticancer drugs induces the protective acute phase response in the human organism. Increased producti-

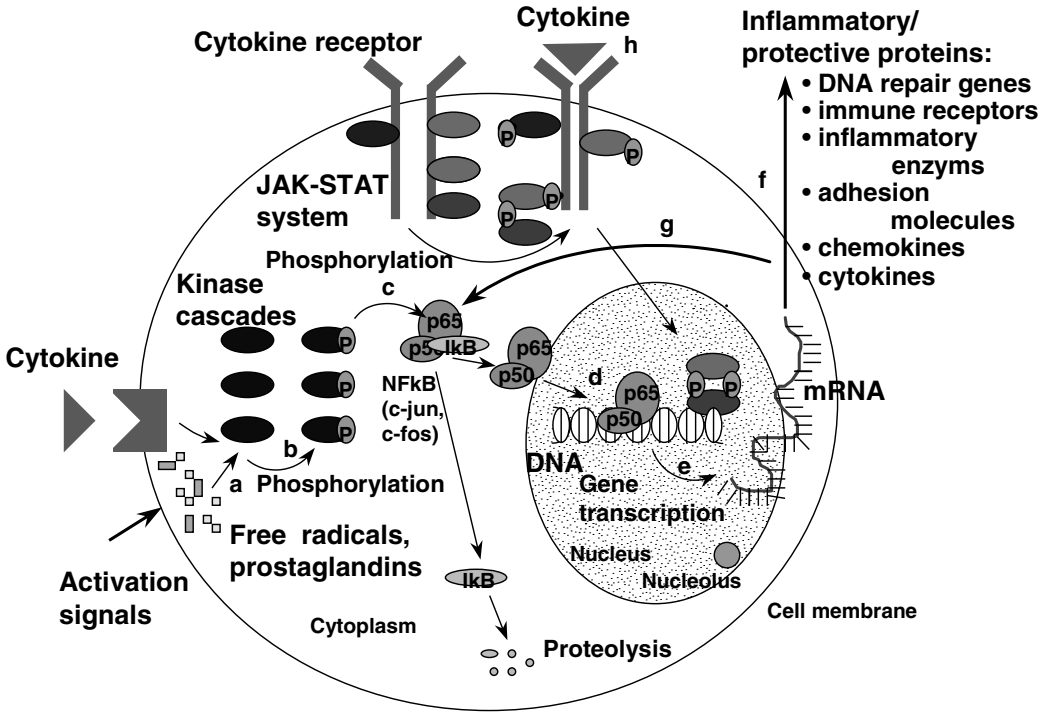

Figure 1. The mechanism of the acute phase response to irradiation at the cellular level. The interaction of ionizing radiation with the target cell induces biochemical changes in the cellular environment and metabolism, generation of free oxygen radicals and arachidonic acid derivatives (a). These changes activate kinase cascades (b) in macrophages and other responsive cells, leading to the activation of transcription factors, such as nuclear factor-κB (NF-κB), c-jun, c-fos (c). Active transcription factors bind to specific recognition elements of DNA in the nucleus and upregulate transcription of genes coding proteins involved in the immune and inflammatory responses (d), leading to an increased formation of messenger RNA (mRNA) and protein (e, f). The proteins released as the end result of this response include cytokines, chemokines, inflammatory enzymes, adhesion molecules, immune receptors and deoxyribonucleic acid repair genes.[7, 9, 10] Some of the cytokines are able to reactivate this cascade through the reactivation of particular transcription factors thus amplifying the response (g). The released cytokines also activate similar response in other cells through binding to the cytokine receptors (h).[3, 7, 9, 11-15] Other mediators of inflammation such as serotonine, histamine, bradykinine, and nitric oxide, and other acute phase proteins are also released. All of this leads to clinical inflammatory manifestations, both at the local level with vasodilation, erythema, edema and pain, and at the systemic level with various behavioral, biochemical and nutritional changes. A part of this response is the release of mediators that attenuate it and cause it to resolve.[3, 6, 11]

on of inflammatory cytokines induced by various anticancer agents has been demonstrated both in vitro and in vivo (Table 1). Chemotherapy-induced immune system activation in vivo is known especially from allogeneic bone marrow transplantation (BMT); conditioning regimens including total body irradiation and high-dose chemotherapy can contribute to the activation of host immune cells with inflammatory cytokine release and upregulation of adhesion molecules. [69, 70]

It is highly probable that a wide range of other known cytokines, chemokines, adhesion molecules and other mediators of inflammation not studied in this model so far are released in response to ionizing irradiation and chemotherapy.

Table 1. Cytokine and adhesion molecule expression in irradiated tissues

Mediator	Irradiated tissue or cell type – animal model	Irradiated human tissue or cells	Induced by CT in vitro	Induced by CT in vivo
TNF-alpha	m. macrophages, lung cells, in vitro (16-19) m. lung, BM, spleen, brain, in vivo (17-21) m. BMT model, serum (22)	PB MNCs, in vitro (23-25) BAL cells, in vivo (25) serum, BMT pts. (26)	paclitaxol, CTX, doxorubicine (MNCs) (55-57)	bleomycine (lung) (58) CTX (+TBI), CTX+busulfan (serum, BMT CR) (59)
IL-1 alpha or beta	m. spleen cells, in vitro (27) m. lung, BM, spleen, small gut, brain, in vivo (18, 20, 21, 27-30) m. BMT model, serum (22)	lung macrophages, in vitro (31) serum after brain irradiation, in vivo (32)	paclitaxol (MNCs) (55, 56)	CTX (+TBI, BMT CR; serum) (60)
IL-2			doxorubicine (MNCs) (61, 62)	
IL-2r		PB lymphocytes, in vitro (33)		
IL-4	m. lung, in vivo (34)			
IL-6	m. BM, spleen, in vivo (20) m. BMT model, serum (10)	macrophages, epithelial cells, lung fibroblasts, in vitro (35-37)	MTX, ARA-C (MNCs) (63)	
IFNgamma				CTX (serum, BMT CR) (65, 66)
TGF-beta	m. lung, small gut, liver, in vivo (17, 29, 38-40) pig skin, in vivo (41)	PB MNCs, in vitro (23) colon, small gut, in vivo (42, 43)	5-FU (fibroblasts, ECs) (64)	bleomycine, CTX (lung) (67,68) CTX (+TBI, BMT CR; serum) (60)
PDGF	m. small gut, in vivo (29)	MNCs, BAL cells, in vitro (25)		
IGF-I		MNCs, in vitro (25)		
FGF	bovine ECs, in vitro (44)			
EGF		serum after brain irradiation, in vivo (32)		
PAF		saliva, in vivo (45)		
ICAM-1	m. lung, brain, in vivo (46-48)	ECs, in vitro, in vivo (46, 49-51) skin, in vitro (49, 52)		
VCAM-1		skin, ECs, in vitro (52)		
E-selectin	m. lung, in vivo (47)	ECs, skin, in vitro (51-54)		
P-selectin	m. lung, in vivo (47)			

Abbreviations: CT, chemotherapy; TNF, tumor necrosis factor; m., murine; BM, bone marrow; BMT, bone marrow transplantation; PB, peripheral blood; MNCs, mononuclear cells; BAL, bronchoalveolar lavage; pts., patients; CTX, cyclophosamide; TBI, total body irradiation; BMT CR, bone marrow transplantation conditioning regimen; IL, interleukin; MTX, methotrexate; ARA-C, cytosinarabinoside; IFN, interferon; GM-CSF, granulocyte macrophage-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; TGF, transforming growth factor; 5-FU, 5-fluorouracil; ECs, endothelial cells; PDGF, platelet-derived growth factor; BAL, bronchoalveolar lavage; IGF-I, insulin-like growth factor-I; FGF, fibroblast growth factor; EGF, epidermal growth factor; PAF, platelet-activating factor; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

Role of cytokines in pathogenesis of radiotherapy- and chemotherapy-induced side effects

A simplified list of immune effects of cytokines and adhesion molecules is involved in Table 2.

A variety of changes in normal tissues are induced by ionizing radiation, depending on the total dose, fractionation schedule, and volume treated.[9] Most normal tissue effects can be attributed to cell killing (cytokines mediate repair processes here); some cannot, though. For instance, the nausea and vomiting that can occur within hours after irradiation of the upper abdomen; the acute edema or erythema that results from radiation-induced acute inflammation and associated vascular leakage; the fatigue in patients receiving irradiation to a large volume, especially within the abdomen; the somnolence and headache after cranial irradiation. These are most likely mediated by radiation-induced inflammatory cytokines.[79] Similar symptoms can be observed after chemotherapy. Nausea, vomiting and fever occurring immediately after chemotherapy and the associated anorexia and fatigue are likely to be also mediated by inflammatory cytokines, such as TNF-α, IL-1, and IL-6. Similar symptoms are associated with infectious diseases; immune response is nonspecific.[3]

The hematotoxicity of chemotherapy and radiotherapy has been generally attributed to the direct damage of rapidly dividing hematopoietic progenitor cells.[79, 80] However, several apparently physiological inhibitors of hematopoesis have been identified that directly or indirectly suppress the proliferative response of progenitor cells to stimulating cytokines; these include TGF-β, macrophage inhibitory protein-1α (MIP-1α), TNF-α, and interferon-γ (IFN-γ).[81-84] Irradiation can induce IFN-γ, TGF-β and TNF-α release, and these and other cytokines might be responsible for hematopoiesis suppression after local irradiation not involving large volumes of bone marrow.[66]

Cell-mediated immune response plays a key role in the pathogenesis of the so-called "anemia of chronic disease".[85] This response may also be involved in the pathogenesis of chemotherapy- and radiotherapy-induced anemia. TNF-α and IL-1 reduce proliferation of erythroid progenitor cells by exerting either a direct inhibitory effect or an indirect effect via the action of IFN-α or IFN-β.[86] TNF-α, IL-1 and IL-6 are able to induce hypoferremia by increasing iron uptake into monocytes/macrophages and synthesis of ferritin, thus contributing to efficient storage of the acquired iron;[87, 88] IFN-γ and IL-2 enhance strongly the expression of the transferrin receptor, the essential protein for iron uptake.[89, 90] Iron deprivation enhances the activity of cytokines such as IFN-α or TNF-α and cytotoxic effects of macrophages in order to produce a protective response as efficient as possible. However, hypoferremia reduces hem synthesis in erythroid progenitor cells.[85]

Proinflammatory cytokines are released immediately after CNS irradiation. The basis of demyelination is the interplay of cytokines between endothelial cells, oligodendrocytes, astrocytes and microglia.[21] The disruption of the endothelium leads to the infiltration of lymphocytes into the tissue and initiation of immunologic mechanisms involved in the pathogenesis of encephalopathy and myelopathy.[21, 28, 91, 92]

Apoptosis, i.e. programmed cell death, is a common mechanism of cell death in response to ionizing radiation and anticancer drug exposure.[93-95] The transmembrane forms of TNF-α and TGF-β released by peripheral blood mononuclear cells have been shown to be involved in the radiation-induced apoptosis of the endothelial cells.[24, 72] Basic fibroblast growth factor (FGF), on the other hand, protects endothelial cells from radiation-induced apoptosis in vitro.[93] The induction of apoptosis is co-responsible for normal tissue damage by irradiation and probably by chemotherapy, too.

Table 2. Biologic effects of cytokines and adhesion molecules.

Mediator	Effects
TNF-alpha IL-1, IL-6	principal proinflammatory cytokines with profound effects on the processing of the acute phase response (1, 11); activation of neutrophils, T-, B-lymphocytes, macrophages, ECs, fibroblasts; induction of cytokines and other inflammatory protein release, upregulation of AM expression, activation of the hypothalamus-hypophysis-adrenal gland synthesis, TNF-alpha induced apoptosis in ECs (1, 69, 71-73)
IL-2	activation of lymphocytes and monocytes (74)
IL-4	growth factor of B-lymphocytes; inhibition of the release of mediators of inflammation (1)
IFN gamma	stimulation of phagocytic abilities of macrophages, differentiation of T-lymphocytes, cytotoxic efects (1)
TGF-beta	suppression of the inflammatory response, stimulation of fibroblast proliferation (40)
PDGF, FGF, IGF-I	stimulation of proliferation of fibroblasts (25, 38)
G-CSF, GM-CSF, M-CSF	hematopoietic growth factors playing a pivotal role in regulation of BM progenitor cell proliferation (75)
EGF	stimulation of epithelial proliferation, and differentiation (76)
PAF	involved in transmigration of leukocytes into the site of inflammation in cooperation with adhesion molecules; mediator of angiogenesis induced by inflammatory cytokines (77)
SCF	stimulation of hematopoietic stem cells (78)
AMs of the immunoglobulin family (e.g. ICAM-1, VCAM-1)	mediate firm adherence of leukocytes to ECs with subsequent extravasation (7)
Selectins (E-, P-selectins)	mediate loose contact between leukocytes and ECs, i.e. leukocyte rolling (7)

Fibrosis is a delayed result of radiation- and chemotherapy-associated tissue damage. It represents a reparation process at the time when the damaging insult does not act on the tissue.[79] Fibrosis is more than a mark of tissue damage; it is damaging in itself.[96] In association with fibrosis development, increased expression of TGFs-β have been found in the irradiated animal lung, liver and skin tissue and in the lungs of bleomycin- and cyclophosphamide-treated mice.[17, 38-41, 60, 67, 68] Their expression is increased both in early and late stages of tissue reaction.[41] TGFs-β have chemoattractive effects on fibroblasts and inflammatory cells and promote cell proliferation; they regulate expression, synthesis and storage of components of extracellular matrix.[40, 97] Other cytokines, such as TNF-α, IL-1, IL-4, PDGF, FGF, insulin-like growth factor-I (IGF-I) are also likely to play an important role in fibrosis development due to fibroblast stimulation.[18, 25, 29, 34, 38]

Proinflammatory cytokines and adhesion molecules are involved in the pathophysiology of BMT-related complications. In an experimental model, the intensification of the conditioning regimen by increasing the total body irradiation dose results in an increased graft-versus-host disease severity. Total body irradiation and allogeneic immune cells appear to act synergistically to damage the gastrointestinal tract, thereby permitting an increased translocation of bacterial endotoxin (lipopolysaccharide) into the systemic circula-

tion. As total body irradiation increases macrophage and endothelial cell priming to lipopolysaccharide resulting in higher systemic levels of inflammatory cytokines, adhesion molecules, and probably other mediators of inflammation, an increase in the severity of graft-versus-host-disease results.[22, 50, 60, 69]

Conclusions

Although the interpretation of cytokine concentrations has to be made with caution with respect to the used method of analysis,[98] there is no doubt that cytokines are released in response to ionizing radiation and chemotherapy. They are involved in the development of side effects of these treatment modalities-that, however, have a protective role in the organism and tissues.

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Efficacy of weekly trastuzumab and paclitaxel in the treatment of women with HER-2/neu overexpressing metastatic breast cancer. The impact of taxane free interval on treatment outcomes

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Purpose. *Trastuzumab is known as an active agent in HER2/neu overexpressing metastatic breast cancer. In the prospective study we investigated efficacy, safety and toxicity of trastuzumab and paclitaxel in metastatic breast cancer progressing on previous therapy.*

Patients and methods. *We accrued 17 patients with histologically confirmed breast cancer, Karnofsky performance status at least 60 %, median age 50 (36-66), pretreated with at least two regimens. HER-2/neu expression was tested by HercepTest (r) (DAKO) in all 17 patients. Fifteen specimens were 3+ positive and 2 specimens 2+ positive. All patients except one were pretreated with taxanes. Taxane free interval (TFI) was defined as a time from last taxane administration until the beginning of the study for every enrolled patient. TFI longer than 1 year was found in 7 patients. TFI shorter than 1 year was observed in 9 patients. Trastuzumab was given 4 mg/kg i.v. as a loading dose followed by 2 mg/kg i.v. weekly. Paclitaxel was given 80 mg/m^2 i.v. weekly until disease progression or unacceptable toxicity. We assessed the response rate (RR), the time to progression (TTP), the survival (OS) and toxicity.*

Results. *In the intent to treat population we found objective response in 10 patients (59 %), including 2 complete responses (CR). In the subset with TFI > 1 year we observed response in 4 cases including 1 CR (RR 57 %). In TFI ≤ 1 year subgroup we observed response in 6 cases also with 1 CR (RR 67 %). TFI was not statistically significant for response ($p < 0,4349$). Median TTP is 6 month with 4 patients remaining progression free. Patients with TFI > 1 year tend to have longer TTP ($p = 0,0201$). Median OS has not been reached with 10 patients surviving. We administered 599 cycles including 473 cycles of trastuzumab and paclitaxel with no dose adjustment. One patient developed hypersensitivity reaction on the first trastuzumab infusion and was excluded from study. The most common toxicity was trastuzumab infusion related pyretic reaction observed in 6 patients. Dose limiting adverse effect which led to the treatment discontinuation was cardiotoxicity. Ejection fraction decline grade 2 occurred in 1 patient and grade 3 also in 1 patient. Six patients experienced grade 3 neuropathy. There were observed 1 episode of grade 4 neutropenia and grade 3 anemia. We noted 4 episodes of grade 3 infection without neutropenia. Grade 3 elevation of liver function tests occurred in 6 patients with no need of dose reduction. There were observed 1 episode of grade 3 hyperglycemia and 1 episode of grade 3 weight gain. Other grade 3 or 4 toxicity was not detected.*

Conclusions. *Trastuzumab and paclitaxel have shown activity and good tolerability in HER-2/neu overexpressing metastatic breast cancer patients. Tumor response in 10 responding taxanes pretreated patients was independent on TFI, but patients with longer TFI tend to be longer progression free.*

Key words: breast neoplasms – drug therapy; paclitaxel; treatment outcome; trastuzumab

Introduction

The HER-2/neu (c-erbB-2) oncogene encodes a 185 kD transmembrane protein with tyrosine kinase activity. This glycoprotein belongs to the family of epidermal growth factor receptors.[1,2] Overexpression of HER-2/neu was found in several solid tumors including breast cancer, lung cancer, prostate cancer, ovarian, gastric and pancreatic cancer.[3,4] In breast cancer overexpression of HER-2/neu has important biological consequences. In preclinical studies HER-2/neu was associated with higher tumorigenicity and metastatic potential.[5,6] HER-2/neu overexpression is considered as a negative prognostic factor in women with breast cancer and in several studies was confirmed the correlation with shorter disease free survival, overall survival and more aggressive tumors.[7,8] HER-2/neu receptor creates homodimers or heterodimers with other members of epidermal growth factor receptor family (EGFR, HER-3, HER-4) on the cell surface which leads to triggering a cascade of growth signals. Studies performed with viral ligands suggest that HER-2/neu evolved as ligandless receptor[9] or this ligand has not been identified so far. The most common and most potent association occurs between HER-2/neu and HER-3. Interestingly, HER-3 has no intrinsic tyrosine kinase activity and cannot respond to ligand binding unless associates with another receptor, such as HER-2, which provides the intracellular signaling.[10]

Some level of HER-2/neu overexpression is found in 25 %-30 % breast cancers with HER-2/neu gene amplification detected in 95 % of the specimens.[7, 11, 12] There are many possible ways to test for HER-2/neu overexpression in tumor cells. Immunohistochemistry measuring protein expression on the cell surface is widely practiced by pathologists around the world and is fast and relatively cheap. Fluorescence in situ hybridization (FISH) measuring gene amplification is easily reproducible but expensive and not widely available. In general, there is a good agreement between the testing strategies. However, there are cases of immunohistochemical "positive" tests with no evidence of gene amplification. There are conversely cases of genetic amplification without increased surface expression.[13] Currently used scoring system for immunohistochemistry has the scale from 0 to 3+. Results 0 and 1+ are understood as negative and 2+ and 3+ as positive.14 Nowadays, there is trend to find only 3+ results as positive because in this subgroup is a concordance with FISH over 75 %[15, 16].

In 2+ subgroup the level of agreement reaches only 24%-39% with significantly lower response rates when treated with monoclonal antibody directed against this protein.[15-17] The definition and standardization of optimal HER-2/neu assay is still in a process.

Using the specific humanized anti-HER-2/neu monoclonal antibody trastuzumab (Herceptin; Roche ®) we can block the activity of HER-2/neu protein and stimulate antibody dependent cellular cytotoxicity.[18] Trastuzumab demonstrated activity in clinical trials in women with HER-2/neu overexpressing metastatic breast cancer as a single agent achieving response rates ranged from 12 % to 27 %.[19-21] Clinical trials based on preclinical evidence of synergy with many chemotherapeutic agents have been conducted. Trastuzumab was combined with cisplatin,[22] or paclitaxel,[16] or vinorelbine[23] in phase II studies with higher response rates than expected for chemotherapy alone. The pivotal phase III trial compared trastuzumab + chemotherapy to chemotherapy alone (either doxorubicin, cyclophosphamide or paclitaxel). Data indicated that trastuzumab in combination with

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chemotherapy produced significantly increased time to progression, response rate and overall survival. Of particular note is that addition of trastuzumab to paclitaxel therapy more than doubled median time to progression and almost doubled the response rate.[24] Regimens with combination of chemotherapy and trastuzumab were generally well tolerated. Pyretic reaction following first trastuzumab infusion occurred in 40 % of patients. Some degree of cardiac dysfunction was observed in 27 % of patients treated with trastuzumab plus doxorubicin and cyclophosphamide which excluded this combination from further clinical use. In trastuzumab plus paclitaxel arm such events were observed in 12 % of patients and only in 2 % were serious.[24] However, trastuzumab plus paclitaxel seems to be a very potent combination, the vast majority of patients in our study were treated with another taxane, docetaxel, in the first or second line treatment. It needs to be clarified whether docetaxel pretreatment and time from the last docetaxel administration have any significant impact on treatment outcomes.

Patients and methods

Eligibility

Women with histologically confirmed metastatic breast cancer overexpressing HER-2/neu were eligible for the purpose of this study. Patients had to be from 18 to 75 years old, with performance status at least 60 % according to the Karnofsky scale. All patients signed written informed consent. Patients were pretreated with two or more regimens for metastatic disease. In case of early recurrence after adjuvant chemotherapy (less than 12 month) also patients pretreated only with one regimen for metastatic disease were eligible. All patients were previously treated with antracyclines (mainly in the adjuvant setting) and all except one with taxanes. Any hormonal treatment except LHRH analogs had to be discontinued before study entry. Laboratory criteria included absolute neutrophile count (ANC) > 1 000/ul, hemoglobin > 80 g/l, platelets > 100 000/ul, adequate hepatic and renal function. Left ventricular ejection fraction had to exceed 50 % with exclusion of patients with history of serious cardiac disease. Patients with clinically unstable metatastases to the brain were not allowed to enter the study. Patients were ineligible if they had a history of other malignancy (except carcinoma in situ of the cervix or nonmelanoma skin carcinoma). Women with childbearing potential had to use reliable contraception while on study and had a negative pregnancy test before entering the study. Baseline evaluation included a complete physical examination, history, complete blood count with differential and platelet count, chemistry, echocardiogram, lesion measurement as appropriate for disease assessment. Her-2/neu status was determined using rabbit 4D5 antihuman HER-2/neu polyclonal antibody (HercepTest).

Treatment

Trastuzumab was administered 4 mg/kg intravenously over 90 minutes as a loading dose with subsequent weekly doses 2 mg/kg over 30 minutes. Paclitaxel 80 mg/m^2 was administered intravenously over 60 minutes the day after trastuzumab loading dose and subsequently the same day after trastuzumab infusion. The treatment was delivered in the outpatient clinic of our department. Treatment was administered every week until disease progression or unacceptable toxicity. Paclitaxel could have been discontinued due to toxicity with further administration of trastuzumab alone until disease progression. Routine premedication before paclitaxel infusion consisted of 8 mg of dexamethasone intravenously (IV), 100 mg cimetidine or 20 mg famotidine IV and 1 mg clemastine IV. Pacli-

taxel was omitted or discontinued for hematologic toxicity (ANC < 1000/ul, platelets < 100000/ul), peripheral neuropathy grade 3 and higher.

Response and toxicity evaluation

Complete blood count was obtained every week and every other week when paclitaxel was discontinued. Serum biochemistry was repeated every four weeks. Echocardiography was performed at least every 16 weeks and at any other time if necessary. Toxicity was graded according to Common Toxicity Criteria National Cancer Institute Version 2.0.

The response was evaluated every three month. The same method as at baseline was used throughout the study. Complete response was defined as a complete disappearance of all signs of tumor confirmed after 4 weeks or later. A partial response was defined as a more than 50 % reduction in the sum of products. Progressive disease was defined as 25 % or bigger increase in the sum of products. All other cases were evaluated as a stable disease.

Immunohistochemical analyses

All specimens of either primary or metastatic tumor were tested for overexpression of HER-2/neu with polyclonal rabbit antihuman antibody (HercepTest DAKO®). We used widely accepted scale when score 3+ is strongly positive, score 2+ is moderately positive, score 1+ means weak positivity, and score 0 is negative. Only patients with 3+ and 2+ results were eligible for protocol. No FISH analyses were performed.

Statistical methods

The primary endpoint of this trial was the overall response to the regimen combining trastuzumab and paclitaxel. The time to progression (TTP) was defined as a time from study entry to progression. Overall survival (OS) was defined as a time from study entry to death. The median time to progression and median overall survival was estimated by the Kaplan-Meier method. TTP was censored in the following circumstances: patient was still receiving treatment without evidence of progression, patient died of unknown cause without evidence of clinical deterioration due to breast cancer and patient discontinued treatment for any reason without evidence of clinical deterioration due to breast cancer before discontinuation. The same criteria was applied for OS. All patients treated with metastatic breast cancer documented at study entry and treated were included in the efficacy intent-to-treat population. Safety analysis included all patients received at least one dose of study drug.

Results

Efficacy data

Between July 1999 and January 2001, 17 eligible patients were enrolled in our institution onto this study. The characteristics are listed in table 1. Only two patients had 2+ Herceptest, all other results were 3+. All patients except one were pretreated with taxanes for metastatic disease (14 with docetaxel and 2 with paclitaxel administered every 3 weeks). Patients were stratified according to the taxane-free interval to two groups: TFI > 1 year; TFI ≤ 1 year.

Altogether 599 cycles of treatment including 473 cycles of trastuzumab plus paclitaxel were delivered. The median number of treatment cycles per patient was 33 (1-78). In one case only first dose of trastuzumab was given with subsequent severe hypersensitive reaction. This patient could not have been evaluated for response. There were no principal protocol deviation. Paclitaxel was discontinued or omitted due to toxicity in 11 pati-

Table 1. Patient characteristics (n = 17)

Characteristics		Patients No.	Patients %
Age			
Median	50		
Range	36-66		
Prior chemotherapy			
2 prior regimens		6	35
≥ 3 prior regimens		11	65
No. of metastatic sites			
1		7	41
2		4	24
≥ 3		6	35
Visceral metastases		11	64
Taxane free interval			
> 1 year		7	41
≤ 1 year		9	53
not pretreated with taxanes		1	6
IHC HER2/neu (Herceptest)			
3+		15	88
2+		2	12
IHC ER			
ER +		4	24
ER -		11	64
Unknown		2	12

Table 2. Response to therapy

Response	Patients No.	Patients %
Overall response	10	59
Complete response	2	12
Partial response	8	47
Stable disease	2	12
Disease progression	4	23
Nonassessable	1	6
Response in patients with TFI ≤ 1 year	6	67
Response in patients with TFI > 1 year	4	57
Response in patients with 3+ IHC	10	67

ents with permanent discontinuation in 6 patients.

Response data are listed in Table 2. There was 2 CRs and 8 PRs with an objective response rate 59 % in the intent-to-treat population. Two patients had stable disease for at least 24 weeks and 4 patients progressed on therapy. The first CR occurred in 53 years old woman with infiltration of soft tissues of chest wall, and the second CR occurred in 36 years old woman with liver involvement. The first CR was maintained for 47 weeks and the second CR was still maintained at the time of analysis (45 weeks from the first documentation). One patient with PR after 16 cycles was referred to surgery for removal of residual disease in contralateral breast. She remains disease free at the time of analysis. In the subgroup with TFI > 1 year was observed 3 PRs and 1 CR. In the subgroup with TFI ≤ 1 year

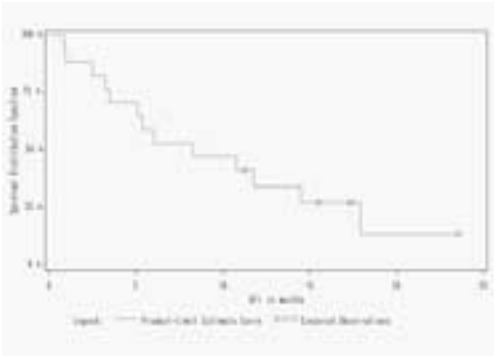

Figure 1. Time to progression in month.

were achieved 5 PRs and 1 CR. TFI does not have any impact on the overall response ($p < 0,4349$).

The median time to tumor progression in the intent-to-treat population was 6 month (range: 1-23,5) (Figure 1). At the time of analysis 4 patients (3 with TFI > 1 year 1 with TFI ≤ 1 year) remained free of progression. Patients with TFI > 1 year tended to stay longer progression free ($p = 0,0201$).

The median survival was not reached at the time of analyses when 10 patients were alive. The 1-year survival in the intent-to-treat population was 53 % and the 2-year survival 12 %.

Safety and toxicity data

All 17 patients were evaluated for toxicity (Table 3). In total there were delivered 599 cycles including 473 cycles of paclitaxel plus trastuzumab with no dose adjustments to 17 patients. The median number of cycles per patient was 33 (range: 1-78). Paclitaxel was omitted or discontinued in 11 patients. Paclitaxel toxicity, mainly neurotoxicity, led to permanent discontinuation of this drug in 6 patients. The most common adverse event was infusion related pyretic reaction after the first trastuzumab infusion in 6 patients (35 %). One patient experienced serious hypersensitivity reaction with dyspnea, shortness of breath and hypertension when receiving first trastuzumab infusion. This event led to treatment discontinuation. The dose limiting adverse effect was also cardiotoxicity. Ejection fraction decline grade 2 occurred in 1 and grade 3 in 1 patient. The treatment was discontinued in both cases when one patient was in CR and one in PR with no further tumor regression. Hematological toxicity was very modest. We noted only 1 episode of grade 4 neutropenia and 1 episode of grade 3 anemia. No growth factors were administered and only 3 units of blood transfusion were gi-

Table 3. Toxicity profile of weekly trastuzumab and paclitaxel

		NCI Grade (% of patients)		
Toxicity		2	3	4
Neutropenia		-	-	1 (6 %)
Leucopenia		3 (18%)	-	-
Anemia		1 (6%)	1 (6%)	-
Neuropathy		2 (12%)	6 (35%)	-
Cardiotoxicity		1 (6%)	1 (6%)	-
Infection		5 (29%)	4 (24%)	-
Hypacusis		2 (12%)	-	-
Edema		4 (24%)	-	-
Nausea/Vomiting		1 (6%)	-	-
Heartburns		1 (6%)	-	-
Onycholysis		1 (6%)	-	-
Weight gain		6 (35%)	1 (6%)	-
Transaminitis		3 (18%)	6 (35 %)	-
High glucose		-	1 (6%)	-
Infusion related reaction:	6 (35%)			

ven. There were observed 3 episodes of grade 3 infection without neutropenia treated with antibiotics with no further complications. Grade 3 elevation of liver function tests occurred in 6 patients with no need of dose reduction. Six patients experienced grade 3 neuropathy, which led to paclitaxel discontinuation in 5 patients. Other serious toxicity was very rare. We observed grade 3 weight gain in 1 patient, grade 2 weight gain in 6 patients and 1 episode of grade 3 hyperglycemia. It remains to be answered whether weight gain is related either to dexamethasone used as a premedication or to study drugs. Other toxicity was only marginal (Table 3).

Discussion

At the time we started to accrue patients to this trial there were no phase II or III data published about the efficacy of trastuzumab plus weekly paclitaxel. We assumed sufficient efficacy based on results of pivotal trial combining trastuzumab with chemotherapy.[24]

This pivotal, multicentre phase III trial randomized 469 HER2 positive (2+, 3+) previously untreated metastatic breast cancer patients either to receive chemotherapy alone or chemotherapy plus trastuzumab. Four arms were designed as follows: AC every 3 weeks alone, AC plus trastuzumab weekly, paclitaxel every 3 weeks alone, or paclitaxel plus trastuzumab. The addition of trastuzumab to chemotherapy almost doubled response rate and prolonged overall survival. When we compare paclitaxel alone to paclitaxel plus trastuzumab the response rate was even more than doubled however the response rate in paclitaxel alone was lower than expected. Most potent has been the combination of AC and trastuzumab but high proportion of cardiac events excluded this combination from further investigation. Nevertheless some grade of cardiac dysfunction occurred in both trastuzumab arms.

In another pivotal phase II trial trastuzumab was administered as a single agent to 222 metastatic breast cancer patients who had failed on previous 1st or 2nd line chemotherapy.[20] The overall response rate in pretreated population was 15 % and 18 % in 3+ population. In the subset with positive FISH response rate was even 20 % and furthermore no FISH negative patient responded to therapy.

The evaluation of HER2 expression was further investigated. There was found 75-89 % concordance between FISH positivity and 3+ result of immunohystochemistry[16,17] but only 24-39 % of patients who were 2+ positive by immunohistochemistry had also positive FISH result. The relative lack of benefit in 2+ population implicated the suggestion that all 2+ results should be confirmed by FISH before the treatment initiation.

The results from preclinical studies showed that trastuzumab has synergic or additive effect with some other drugs including vinorelbine, carboplatin, cisplatin, docetaxel, gemcitabin etc. Interesting data has come up from the trial with weekly trastuzumab and vinrolebine as the 1st, 2nd and 3rd line therapy of metastatic breast cancer with overall response rate exceeding 70 %.[23] Just recently there were published data from trastuzumab and gemcitabine phase II trial.[25] In several ongoing trials is trastuzumab combined with weekly docetaxel or carboplatin plus minus docetaxel.[26]

Because of promising safety and efficacy profile of trastuzumab combinations in the metastatic setting, this novel biologic agent now entered adjuvant breast cancer trials in the United States and Europe. Breast Cancer International Research Group conducts a clinical trial (BCIRG 006) for node positive early breast cancer patients.[27] Other examples of this approach include the National Surgical Breast and Bowel Project clinical trial B-31, the North Central Cancer Treatment Group adjuvant trial 9831 and the Eastern Coopera-

Figure 2. Trastuzumab adjuvant trials.

PTX = paclitaxel; T = trastuzumab; AC = doxorubicin and cyclophosphamide; DTX = docetaxel, CBDCA = carboplatin
q3w = every 3 weeks; w = weekly;
S = surgery; RT = radiotherapy; CT chemotherapy

1) ECOG 2198 (N+)
PTX q3w × 4 + T → AC × 4 → No T
→ T × 52 w
2) Intergroup NCCTG N 9831 (N+)
AC q3w x 4 → PTX w × 12
→ PTX w × 12 → T x 52 weeks
→ PTX w × 12 + T w → T × 40 weeks

3) NSABP B-31 (N+)
AC q3w × 4 → PTX × 4
→ PTX × 4 + T × 52 weeks
4) BCIRG (N+)
á AC × 4 → DTX × 4
á AC × 4 → DTX × 4 + T × 52 weeks
á DTX + CBDCA × 6 + T × 52 weeks
5) BIG HERA
S + CT + RT →T q3w 1 year → T q3w 2nd year
→ observation
→ observation

tive Oncology Group trial 2198 (Figure 2).[26] The Breast International Group conducts an adjuvant trial called HERA which slightly differs from other trials mentioned before because trastuzumab is planned to be administered every 3 weeks (Figure 2).[26,28] Completion of these ongoing trials through collaborative efforts between patients and scientific community will allow us to obtain the results we so eagerly await.

Conclusions

In this small single institution prospective open labeled clinical trial we showed that trastuzumab and paclitaxel is active in the treatment of HER-2/neu overexpressing metastatic breast cancer patients with only limited number of adverse event. As the first group we assessed the effect of this combination with respect to the interval from the last taxane administration – taxane free interval. We did not found any statistically significant correlation between taxane free interval and overall response rate. Nevertheless, patients with longer taxane free interval tended to stay longer progression free.

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review

Natural inhibitors of tumor-associated proteases

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The turnover and remodelling of extracellular matrix (ECM) is an essential part of many normal biological processes including development, morphogenesis, and wound healing. ECM turnover also occurs in severe pathological situations like artherosclerosis, fibrosis, tumor invasion and metastasis. The major proteases involved in this turnover are serine proteases (especially the urokinase-type plasminogen activator/plasmin system), matrix metalloproteases (a family of about 20 zinc-dependent endopeptidases including collagenases, gelatinases, stromelysins, and membrane-type metalloproteases), and cysteine proteases. In vivo, the activity of these proteases is tightly regulated in the extracellular space by zymogen activation and/or controlled inhibition. In the present review, we give an overview on the structure and biochemical properties of important tumor-associated protease inhibitors such as plasminogen activator inhibitor type 1 and type 2 (PAI-1, PAI-2), tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4), and the cysteine protease inhibitor cystatin C. Interestingly, some of these inhibitors of tumor-associated proteases display multiple functions which rather promote than inhibit tumor progression, when the presence of inhibitors in the tumor tissue is not balanced.

Key words: cystein protease; cystatin; matrix metalloproteinase; serine protease; serpin; tissue inhibitor of matrix matalloproteinase

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PAI-1 and PAI-2, natural inhibitors of the urokinase-type plasminogen activator (uPA)

The tumor cell surface-associated urokinase-type plasminogen activator system consists of the serine protease uPA, its receptor uPAR, and the inhibitors PAI-1 and PAI-2. Various normal and cancer cells produce uPA as a single-chain pro-enzyme (pro-uPA) that is proteolytically converted to an active two-chain form, *e.g.* by plasmin or cysteine proteases such as cathepsins B and L.[1] Binding of uPA to uPAR (CD87) focuses the proteolytic activity on the tumor cell surface. In addition to uPAR, tumor cells also express binding sites for plasmin(ogen). uPAR-bound uPA efficiently converts tumor cell-associated plasminogen into plasmin, an active serine protease with broad substrate specificity. Plasmin degrades a variety of components of the extracellular matrix (*e.g.* fibrin, fibronectin, or laminin) and activates several matrix metalloproteases that additionally break down certain macromolecules of the extracellular matrix such as fiber-forming collagens and/or the basement membrane protein collagen IV.[2,3]

The uPA/uPAR system is under the control of the plasminogen activator inhibitors type 1 (PAI-1) and type 2 (PAI-2) both belonging to the serine protease inhibitor super-family (serpins) and sharing 55 % sequence homology (amino acid identity: 33 %). Their structural similarity was shown by X-ray crystal structures of active mutants of PAI-1 and PAI-2 (Figure 1A, B).[4,5] These two inhibitors interact with uPA and the other plasminogen activator, tPA (tissue-type plasminogen activator), forming 1:1 stoichiometric complexes with the respective target protease. tPA, in contrast to uPA, does not bind to a high-affinity receptor on tumor cell surfaces and, therefore, does not promote tumor cell-associated pericellular proteolysis. Whereas PAI-1 recognizes and inhibits all active forms of the proteases (two-chain uPA as well as single- and two-chain tPA), PAI-2 only acts as an inhibitor for the two-chain forms of uPA and tPA.[6] For inhibition, the surface-exposed reactive center loop (RCL) of PAI-1 or PAI-2 interacts with the reactive site of the target protease. Initially, the P1-P1′-bond of the inhibitor (R346-M347 in the case of PAI-1; R380-T381 in the case of PAI-2) is cleaved and a covalent bond between the hydroxyl-group of the catalytic serine residue of the protease and the carboxyl-group of the P1-residue of the RCL of the serpin is formed (acyl-enzyme intermediate). Upon cleavage of the P1-P1′-bond, the RCL is rapidly inserted into the central β-sheet A as additional β-strand 4A, which leads to the translocation (> 70 Å) of the protease across the plane of β-sheet A of the serpin and the formation of a stable enzyme/inhibitor complex. The structure of PAI-1 or PAI-2 in complex with a target protease has not been solved yet, however, the X-ray crystal structure of another serpin-protease complex (trypsin/antitrypsin) has recently been published.[7] The experimental data confirm the theoretical model for the inhibition of serine proteases by serpins.[8,9] In the trypsin/antitrypsin complex, the structure of the hyperstable serpin is hardly changed, whereas the active site of the protease is massively disordered, which prevents the release of the protease from the complex.[7]

PAI-1 is synthesized as a 402 aa-protein (inclusive an N-terminal signal peptide) and secreted by the cell in a glycosylated form. The mature protein (379 aa; M_r: ≈ 50,000) contains no cysteines and, therefore, no disulfide bridges. Active PAI-1 is meta-stable and spontaneously converts to a latent form (which does not inhibit its target serine proteases) by inserting a major part of its RCL into the central β-sheet A (Figure 1C). The biologically active conformation of PAI-1 (half-life: ≈ two hours) is stabilized by vitronectin (Vn), an extracellular matrix (ECM) and plasma protein (half life of PAI-1 bound to Vn: ≈ four hours).[10] Upon binding of Vn to active PAI-1

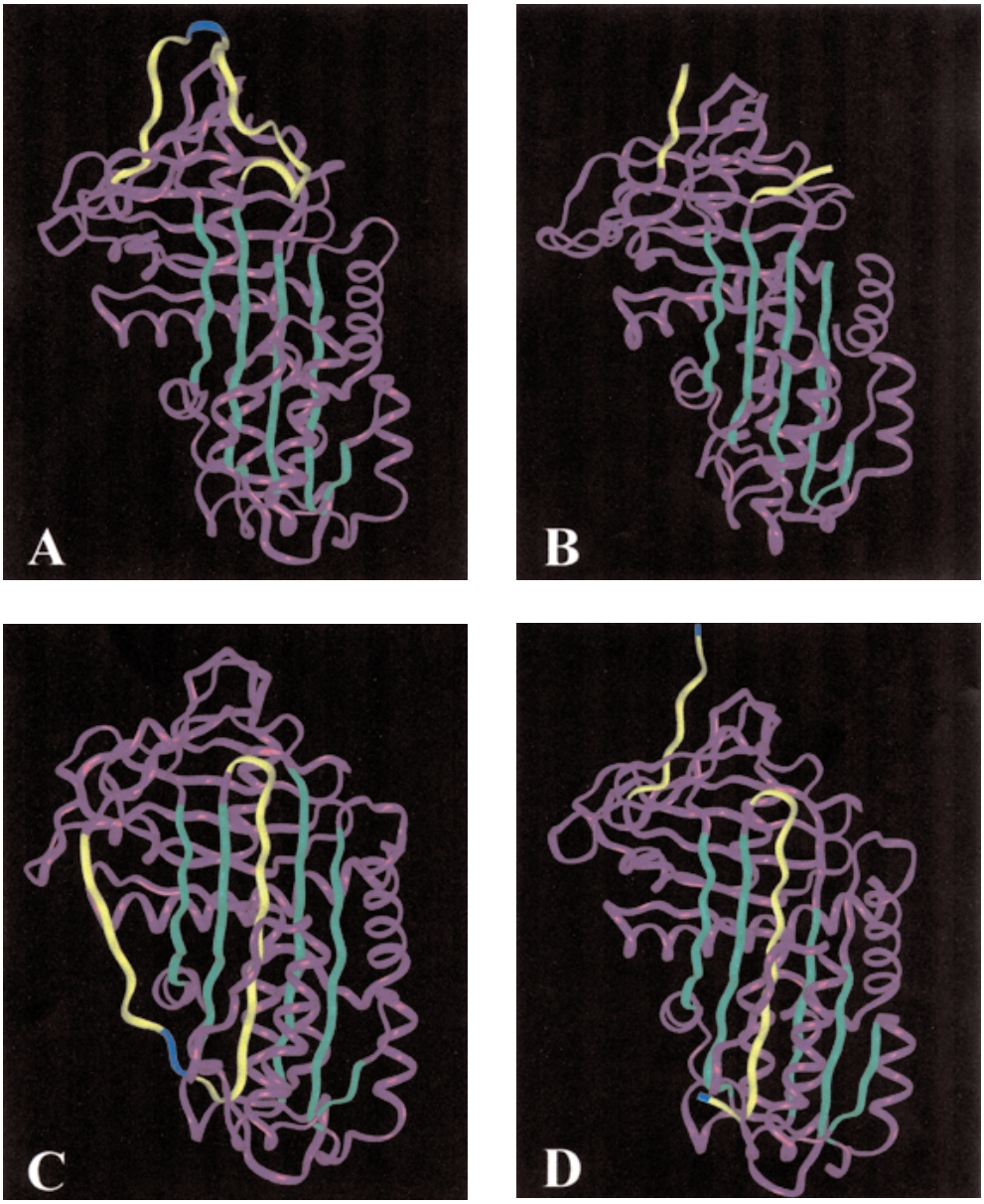

Figure 1. The serpins PAI-1 and PAI-2
Ribbon representation of the tertiary structure of PAI-1 and PAI-2: **(A)** active conformation of PAI-1 (pdb-code: 1B3K), **(B)** active conformation of PAI-2 (pdb-code: 1BY7), **(C)** latent conformation of PAI-1(pdb-code: 1DVN), and **(D)** substrate cleaved form of PAI-1 (pdb-code: 9PAI). The central β-sheet is colored green, the reactive center loop (RCL) is yellow and the localization of the P1 and P1′ residues are blue. In the active conformation of PAI-1 **(A)**, the RCL represents a very flexible loop which is interlaced as an additional β-strand in the latent conformation **(C)**, while in the substrate cleaved form the essential residues P1 and P1′ of the RCL are pulled far apart **(D)**. Because of its flexibility, the localisation of the RCL of PAI-2 was not clearly defined in the X-ray structure and is therefore not depicted in **(B)**. This is also true for several other parts of the molecule. The figures were drawn on the coordinates from the Brookhaven Protein Database using the computer program INSIGHT II (Molecular Simulations Inc.).

structural changes in PAI-1 are induced, providing it with the inhibitory properties against the serine proteases other than uPA and tPA, namely thrombin and activated protein C.[11,12] Recently, alpha(1)-acid glycoprotein has been shown to interact with PAI-1 as well and to stabilize the active form of this inhibitor.[13] In addition to Vn and alpha(1)-acid glycoprotein, PAI-1 interacts with heparin, fibrin, and – when present as a PAI-1/uPA complex – with members of the lipoprotein receptor-related protein (LRP) receptor family.[6] Under certain conditions, when the distortion of the active site of the protease in complex with the serpin cannot keep pace with ester bond hydrolysis, PAI-1 can also be cleaved in a substrate-like manner, and the inhibitor is released from the active protease as the so-called RCL-cleaved form of PAI-1 (Figure D).[14,15]

PAI-2 lacks a cleavable signal peptide and is mainly present intracellularly in a non-glycosylated form (415 aa; M_r: ≈ 47,000). Only a small amount of PAI-2 (≈ 20%) is glycosylated and secreted (M_r: ≈ 55,000). PAI-2 spontaneously forms polymers, very likely by a loop-sheet polymerisation mechanism, in which the RCL of one molecule inserts as an additional β-strand into the central β-sheet of another molecule.[16] The mainly intracellular location of PAI-2 developed a hypothesis that this serpin has some other functions in addition to the inhibition of plasmin generation (which occurs extracellularly). In fact, PAI-2 has been shown to inhibit apoptosis.[17] Furthermore, PAI-2 may function as an antiviral agent and be of relevance in Alzheimer's disease and in some inflammatory reactions.[16,18] The inhibitory effect of PAI-2 depends on both the active site and interhelical loop between helices C and D. This loop has been implicated in transglutaminase-catalyzed cross-linking of PAI-2 to cell membranes.[17] Besides the target proteases uPA and tPA (and transglutaminases), no further intra- or extracellular interaction partners of PAI-2 have been identified so far. In addition to PAI-1 and PAI-2, there are other serpins, *e.g.* proteinase nexin-1, protein C inhibitor, and maspin (a serpin with tumor suppressive activity), which are also capable to inhibit uPA (and tPA) under physiological conditions.[3,19]

PAI-1 and PAI-2 in cancer

In a variety of malignancies such as breast, ovarian, esophageal, gastric, colorectal or hepatocellular cancer, a strong clinical prognostic impact has been attributed to components of the uPA-system, especially PAI-1 and uPA that are statistically independent factors with the capacity to predict the probability of disease-free and/or overall survival.[1,20-23] In general, elevated tumor antigen levels of PAI-1 and/or uPA are associated with poor disease outcome and are conductive to tumor cell spread and metastases. The clinical finding that the uPA inhibitor PAI-1 does not exert a protective function but is an indicator of bad prognosis for cancer patients appears rather striking at first sight. However, additional functions of PAI-1 have been described, which strongly suggest an involvement of PAI-1 in the tumor promoting processes, especially in the modulation of tumor cell attachment or migration and in angiogenesis.[24,25] As the binding sites of PAI-1 and uPAR on the ECM protein Vn overlap, PAI-1 is able to regulate uPAR-mediated cell adhesion by competing with uPAR for binding to Vn. Furthermore, PAI-1/Vn-interaction also affects integrin-mediated cell adhesion to Vn by sterically blocking integrin binding to the RGD (Arg-Gly-Asp) sequence which is immediately adjacent to the PAI-1 binding site on Vn.[26,27] Malignant murine keratinocytes, transplanted into PAI-1-deficient mice, did not invade the surrounding tissue (local invasion). Additionally, the PAI-1-deficient hosts failed to vascularize the implanted tumor cells. Upon intravenous injection of an ade-

noviral vector expressing human PAI-1 in these tumor-bearing mice, tumor cell invasion and associated angiogenesis were restored.[28] Some recently published data report of plasmin involvement in the assembly of new tumor vessels and indicate that PAI-1 is essential for controlling excessive plasmin proteolysis which would otherwise prevent the formation of these vessels.[25,29] The PAI-1/Vn-interaction may also play a part in tumor neovascularization.[29]

In contrast to the consistent association of high tumor tissue concentrations of PAI-1 (and uPA and uPAR) with poor prognosis, various studies analyzing the prognostic impact of PAI-2 have shown different associations between the PAI-2 levels in tumor tissue and patient survival. On the one hand, high antigen levels of PAI-2 in tumor tissue have been associated with good prognosis in patients with breast cancer, small-cell lung cancer and ovarian cancer, but on the other, with a poor prognosis in colorectal and endometrial cancer.[30,31]

Biochemical properties of the TIMPs, the inhibitors of matrix metalloproteases

In addition to the uPA/plasmin system, there is compelling evidence that matrix metalloproteases (MMPs) also act as key players in the events that underlie tumor dissemination.[32] Tumor and stromal cells produce soluble and cell-surface anchored MMPs, which mediate ECM degradation, release of sequestered latent growth and angiogenic factors, and activation of latent growth factors. The proteolytic activity of MMPs is controlled by the so-called TIMPs.

Currently four members of the TIMP-family (TIMP-1, -2, -3 and -4) are known and characterized.[33] These are small proteins with a molecular weight between 21 kDa and 28 kDa and are secreted by many different cell types. They share common structural features including an N-terminal and a C-terminal subdomain, each stabilized by 3 disulfide bonds. Each TIMP has 12 conserved cysteine residues, contributing to the secondary structure and their ability to inhibit MMPs.[34,35] TIMP-1 is glycosylated (8-9 kDa), TIMP-3 can contain sugar components up to 7 kDa;[36] whereas TIMP-2 and TIMP-4 are non-glycosylated.

The N-terminal domain of the molecules harbors the inhibitory activity which forms a tight 1:1 non-covalent complex with the catalytic center of active MMPs (Figure 2). Binding to latent MMPs occurs in a 1:1 stoichiometry at the C-terminal region of individual MMPs.[37] Recombinant truncated TIMPs containing only the N-terminal domain retain most of their inhibitory activity towards

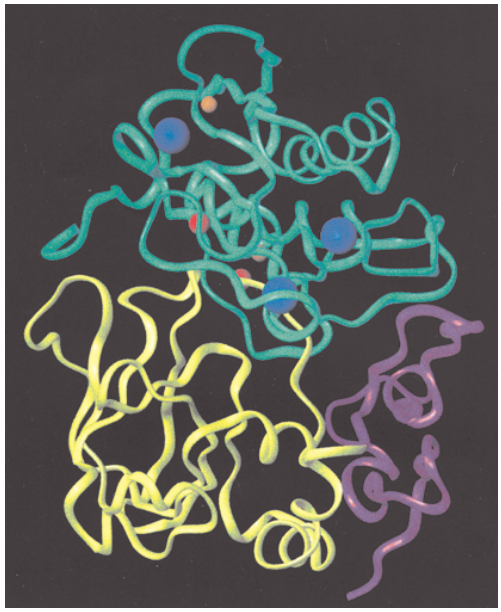

Figure 2. The TIMP-1/MMP-3 complex.
Ribbon representation of the TIMP-1/MMP-3 complex structure (pdb-code: 1UEA): the N-terminal domain of TIMP-1 (residues 2-126) is colored yellow, the C-terminal domain in magenta; MMP-3 is green, catalytic zinc is red, calcium is blue and selenium orange. Only the N-terminal domain of the TIMP-1 molecule performs interactions to the MMP-3 protein in the complex. The figures were drawn on the coordinates from the Brookhaven Protein Database using the computer program INSIGHT II (Molecular Simulations Inc.)

MMPs.[38] The four different TIMPs bind to most subtypes of latent and active forms of MMPs with only minor differences in their inhibitory potential. Variable affinities were elucidated for TIMP-1 and TIMP-2, the former binding to the latent form of MMP-9 with a higher affinity than the latter, while the converse relationship was found for binding to latent MMP-2.[39] High levels of TIMP-2 or -3, but not of TIMP-1 inhibit the activity of MT1-MMP, thereby preventing the latent MMP-2 activation.[40]

The C-terminal domain of the TIMPs is more variable and is involved in the interaction with pro-MMPs.[41,42] Furthermore, this domain might be responsible for the additional biological functions of the TIMPs in proliferation, angiogenesis, and apoptosis.[43,44] These effects are independent of the inhibitory function of TIMPs; however, the mechanisms of these actions are not understood yet.

TIMP-3 is unique because it is unsoluble and tightly bound to the extracellular matrix by its C-terminal domain. Furthermore, TIMP-3 is correlated with the hereditary disease Sorsby`s fundus dystrophy that is caused by a single base pair exchange in the sequence coding for the C-terminal domain.[45]

Modulation of tumor growth and metastasis by TIMP expression in the tumor environment

Recent clinical studies have stated TIMP-1 and TIMP-2 to be rather tumor-promoting molecules, as they were found to be significantly overexpressed in patients with poor prognosis.[46,47] However, it is important to note that, in these studies, the MMP/TIMP ratio was not determined, while the evaluation of either TIMP or MMP expression alone is likely not sufficient for prognostication of malignancies. It is generally accepted that the net proteolytic activity in the tissue is responsible for tumor cell invasion-promoting ECM turnover. Consequently, (gene-) therapeutic intervention by overexpression of TIMPs in the tumor microenvironment is supposed to inhibit ECM degradation and metastasis.[48] The MMPs, the target molecules for the TIMPs, are proteolytically active in the extracellular space. Therefore, in a therapeutic approach, it is not necessary that all tumor cells are transduced by gene therapy vehicles to express elevated levels of TIMPs. It is sufficient that host cells, located at the interface surrounding the primary tumor or the invading cells in the target organ of metastasis, overexpress and secrete TIMPs. The proteolytic balance in the tumor-host environment could thus be shifted in favor of blocking proteolysis, resulting in the inhibition of tumor invasion and metastasis. TIMP-1 transgenic mice have been used to elucidate the feasibility of such an approach and to assess the protective potential of host TIMP-1 on primary tumor growth and metastasis. In the first of these studies, the crossing of transgenic mice that constitutively overexpress TIMP-1 in the liver with transgenics expressing SV40 T antigen, which develop hepatocellular carcinoma, resulted in inhibiting the tumor initiation, growth, and angiogenesis.[49] In another approach, two transgenic mouse lines were used, one overexpressing TIMP-1 (TIMP-1^{high}), and the other expressing the antisense TIMP-1 RNA, leading to TIMP-1 reduction in the tissue (TIMP-1^{low}).[50] TIMP-1 overexpression (TIMP-1^{high}) inhibited tumor growth and spontaneous metastasis of an aggressive T-cell lymphoma, thereby prolonging the survival of mice. Opposite effects occurred in TIMP-1^{low} mice: experimental metastasis assays demonstrated that TIMP-1-compromised livers in TIMP-1^{low} mice showed at least a doubling of metastatic foci and numerous additional micrometastases, indicative of increased host susceptibility.[51] In another experimental setting, experimental metastasis of a fibrosarcoma in the brain could significantly be inhibited in transgenic mice overexpressing TIMP-1.[50] Similar studi-

es with transgenics for TIMP-2, -3, and -4 have not been reported so far. The encouraging results with TIMP transgenic mice have stimulated preclinical gene therapy experiments in mice. Employing adenoviral vectors for TIMP-2 gene transfer to the liver of mice prevented the growth of colorectal metastasis in this organ.[52] Recently, the protection of the host environment was provided by the dramatic overexpression of TIMP-1 due to adenoviral gene transfer, inhibiting the growth of experimental liver metastasis of the T-cell lymphoma (employed in the studies with the transgenic mice mentioned above) and colorectal carcinoma.[53] The systemic increase of TIMP-4 due to intramuscular gene delivery resulted in the inhibition of Wilm's tumor growth,[54] but induced mammary tumorigenesis, most likely due to the anti-apoptotic features of TIMP-4.[55] Gene therapy with vectors encoding TIMP-1 or TIMP-3 to protect the host environment from metastasis has not yet been documented.

The vast literature on the genetic alteration of tumor cells themselves with TIMPs has revealed conflicting data on the usefulness of natural TIMPs in the direct genetic modification of tumor cells. However, these studies indicate that genetic engineering of TIMPs devoid of their additional biological functions (*e.g.* growth factor activity) to increase anti-tumor specificity, might be a useful therapeutic approach.[33]

Cystatins, the natural inhibitors of cysteine proteases

Cystatins comprise single-chain inhibitory proteins that reversibly inhibit the proteolytic activity of cysteine proteases, which are widely distributed in the human body.[56-59] Three types of cystatins are present in vertebrates: type-1 cystatins that are synthesized without a signal peptide and generally present in the cell (cystatin A and B, also named stefin A and B); the secretory type-2 single-domain cystatins (C, D, M/E, F, S, SN, SA) and type-3 multi-domain cystatins (high and low molecular weight kininogens). The type-1 cystatins (approx. 100 aa; M_r: ≈ 11- 12,000) lack both disulfide bridges and carbohydrate groups. Type-2 cystatins (*e.g.* chicken cystatin and human cystatin C) are molecules of about 120 aa (M_r: ≈ 13-14,000) and are characterized by two intrachain disulfide bonds located towards the C-terminus. With the exception of the rat cystatin C, type-2 cystatins are non-glycosylated.[60] Type-3 cystatins encompass three type-2 cystatin-like domains that most probably arose by gene duplications.[61] They contain additional disulfide bonds and are glycosylated.

The secondary structures of chicken cystatin, human cystatin C, and the type-1 cystatins are very similar. A cystatin molecule consists of an N-terminal straight five-turn α-helix ((1) and a five-stranded antiparallel β-pleated sheet (β1 N-terminal and β2-β5 C-terminal), twisted and wrapped around the α-helix.[62] In the case of human cystatin C, no evidence was found for an α-helical conformation of the region unique to the type-2 cystatins (aa T71-L91) that was found in chicken cystatin.[63]

As further demonstrated by crystallographic studies, human cystatin C forms very tight symmetric dimers by a mechanism called three-dimensional domain swapping.[64] Furthermore, higher aggregates may arise through this mechanism in an open-ended way, in which partially unfolded molecules are linked into infinite chains, also found in the brain arteries of elderly people with amyloid angiopathy. An even more severe disease is associated with the L68Q mutant of human cystatin C that destabilizes the monomers and increases the stability of the partially unfolded intermediate causing massive amyloidosis, cerebral hemorrhage, and death in young adults.[63]

Cystatins form equimolar, tight and reversible complexes with papain-like cysteine

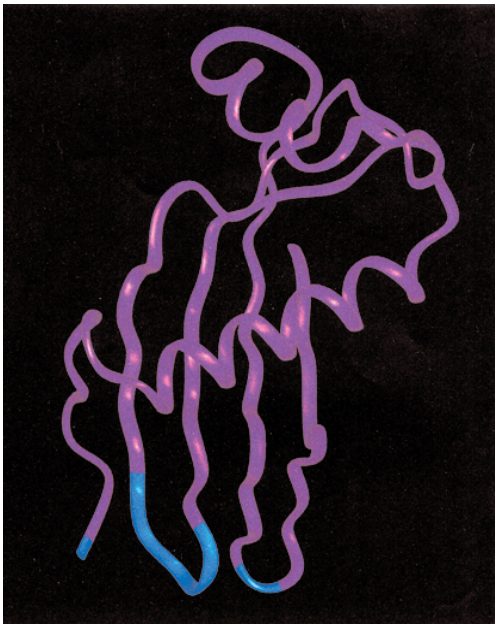

Figure 3. Chicken cystatin.
Ribbon representation of the structure of chicken cystatin (pdb-code: 1CEW). The residues essential for the inhibition of cysteine proteases are distributed on three different adjacent loops and are colored blue. The figures were drawn on the coordinates from the Brookhaven Protein Database using the computer program INSIGHT II (Molecular Simulations Inc.)

proteases.[65] There are three well-conserved regions in the cystatin superfamily that have been implicated in cysteine protease inhibition (Figure 3). These regions are (i) a region near the N-terminus, (ii) a first hairpin loop containing the highly conserved sequence Gln-Xaa-Val-Xaa-Gly, and (iii) a second hairpin loop containing a Pro-Trp pair.[62,66] All three regions contain several hydrophobic residues, indicating that hydrophobic interactions play an important role in the interaction of cystatins with target molecules. Based on structural theory, these three regions penetrate the active site of the enzyme in such a way that the papain active site Cys25 residue is blocked.[62] An additional reactive site in the loop between the α-helix and the first strand of the main β-pleated sheet with its Asn39 residue was detected and shown to be responsible for the inhibition of mammalian legumin by some cystatins.[67]

The role of cystatins in cancer progression

In mammals, cystatins are found in relatively high concentration in biological fluids such as seminal plasma, cerebrospinal fluid, plasma, saliva, and urine. Cystatins A, B, and C are present in high molar concentration in various cells and tissues,[66,68] whereas cystatins D, S, SN, and SA are almost limited to saliva, tears, and seminal plasma.[59,69] Kininogens are major plasma proteins, involved in the tonus regulation of blood vessels and coagulation in addition to their function as cysteine endopeptidase inhibitors.[65]

A large number of normal and pathophysiological processes are controlled by balancing cysteine proteases and their inhibitors. Uncontrolled proteolysis by human cysteine proteases can cause irreversible damage such as inflammatory diseases, neurological disorders, infection, and tumor metastases.[70-74] Cathepsin B, H, and L, primarily lysosomal cysteine proteases, are also important matrix proteases that are involved, together with plasminogen activator and metalloproteases, in cancer invasion by degrading extracellular matrix components.[75-78] Cystatin C is the strongest inhibitor of cysteine proteases, it was therefore most frequently investigated in tumor invasion and metastasis. Tumor-associated expression of cystatin C was at first detected in the ascitic fluid from patients with ovarian cancer.[79] Cystatin C is also increased in the blood of patients with breast cancer,[80] fibrosarcoma,[81] melanoma,[71] colorectal carcinoma,[73] and lung cancer.[82] In these studies, it was reported that the cystatin C content in melanoma, colorectal, and lung cancer patients is associated with the progression of the malignant disease. Furthermore, cathepsin B/cystatin C complexes were found to be less abundant in the

blood of patients with malignant tumors than in healthy controls indicating an imbalance between cysteine proteases and cystatin C in cancer cells.[83] In comparison to normal tissue, a significant decrease of cystatin activity in breast carcinomas compared to normal tissue was noted[80] also in the cerebrospinal fluid and blood from patients with brain tumors.[84] An elevated concentration of the latent (inactive) fraction of cystatins was determined in the blood of patients with head and neck cancer and in the urine of patients with colorectal cancer.[85,86] The significant decrease of the inhibitory activity of cystatins in biological fluids in cancer patients may be taken as a further support to the assumption of an involvement of cysteine proteases in tumor progression and metastasis.[75]

Cystatin C activity is, in fact, correlated to the malignant phenotype as shown by *in vitro* and *in vivo* tumor model systems. Transfection of cystatin C cDNA into B16 melanoma cells led to an inhibition of tumor cell invasion through an artificial matrix barrier *in vitro* and to a significant reduction of the number of lung metastases after the injection into the tail vein of nude mice.[87,88] The inhibitory effect of cystatin C on tumor cell invasion was also demonstrated in *in vitro* Matrigel assays using transfected murine SCC-VII squamous carcinoma cells,[89] ras-transformed breast epithelial cells,[90] human fibrosarcoma, and colon carcinoma cell lines.[81] It is worth to mention that, in cystatin C-deficient mice, a reduction of lung colonization of mouse melanoma cells (expressing cystatin C) was observed.[91] This indicates that an excessively high local cysteine protease activity may inhibit metastatic spread to some tissues. Since other proteases including uPA and matrix metalloproteases are also key molecules in tumor cell metastasis, it is an interesting strategy to modulate the activities of cysteine and other proteases simultaneously.[92]

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Cysteine proteinase inhibitors stefin A and stefin B in operable carcinoma of the head and neck

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Purpose. *To evaluate the significance of cysteine proteinase inhibitors stefins (Stefs) A and B for a treatment decision and prognosis in operable squamous cell carcinoma of the head and neck (SCCHN).*
Patients and methods. *Stefs A and B concentrations were determined immunobiochemically using ELISAs in cytosols prepared from the tumor and adjacent normal mucosa from 91 patients with operable SCCHN. The median follow-up period of patients alive at the close-out date was 5.8 years (range, 5-9.3 years).*
Results. *Stef A concentrations were significantly higher in tumor compared to normal mucosa (P=0.05). When a subgroup with clinically palpable node(s) at presentation was taken into consideration (n = 57), a significant difference in Stef A (P = 0.03) and Stef B (P = 0.02) concentrations between those with negative and positive necks, as determined on histopathological examination, was observed. On the univariate survival analysis, higher Stefs' concentrations turned to be prognostically advantageous. Stef A proved its independent prognostic significance also on multivariate setting.*
Conclusions. *With the capability to differentiate between the pN0- and pN+-stages of the disease in the patients originally presented as node-positive, Stefs A and B could be useful markers when deciding on the extent of neck surgery. In addition, both Stefs proved to be reliable prognosticators for survival in patients with operable SCCHN.*

Key words: head and neck neoplasms; carcinoma, squamous cell; cysteine proteinases inhibitors

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Introduction

Alterations in the balance between cysteine proteinases and their endogenous inhibitors have been postulated to contribute to malignant progression. A large body of literature has been accumulating evidence to suggest that they could be used as prognostic markers in a large spectrum of benign and malignant diseases.[1] However, their prognostic significance in the squamous cell carcinoma of the head and neck (SCCHN) was much less investigated. Our research team conducted the most extensive research in this field and arrived at the conclusion that the cytosolic concentrations of the inhibitors stefins (Stefs) A and B were strongly related to the survival probability.[2] The results on their serum concentrations as well as those related to proteinases were negative in this respect.[2,3]

The present study is a re-analysis of the data on Stefs A and B in patients with exclusively operable disease, exploiting the advantage of longer follow-up. In addition to the prognostic value of Stefs A and B, their significance for a treatment decision was analysed.

Patients and methods

Patients

Ninety-one previously untreated patients with primary operable squamous cell carcinoma of the head and neck entered the study. The routine diagnostic work-up comprised a clinical examination and endoscopy of the aerodigestive tract, chest X-ray, and standard haematological and biochemical tests. In all patients, therapeutic surgery of the primary tumour related to the lesion extension, and neck node dissection were performed. Postoperative radiotherapy was applied in all but nine patients with low-risk disease. The radiotherapy doses were adapted to the disease extent and ranged between 50-66 Gy (median, 56 Gy). Patients were irradiated on a Cobalt-60 unit or a 5-MV linear accelerator, with a daily dose of 1.8-2 Gy, 5 days per week. Tumors were staged after the histopathological examination of surgical specimens (pathological stage) according to UICC TNM classification[4], clinical N-stage before surgery was also recorded. The histopathological grade was defined according to WHO criteria.[5] Clinical features of the patients and their tumors are shown in Table 1.

As of October 31, 2001 (close-out date), 53 patients died: 22 due to the disease recurrence and/or dissemination and 31 due to causes other than the treated malignant disease. Thirty-three patients were alive with no signs of the disease and five patients were lost from follow-up; they were considered to be ineligible for the analysis of survival. The median follow-up period of all eligible patients as calculated from the date of surgery was 4.3 years

Table 1. Patient and tumor characteristics ($n = 91$)

Patients

Age: Median, 58 years (range, 37-72 years)

Sex: Female, 6; Male, 85

Tumors

Site: Oral cavity, 16
Oropharynx, 21
Hypopharyngis, 11
Larynx, 43

pTNM-stage:

	pN0	pN1	pN2	pN3	total
pT1	3	2	2	0	7
pT2	13	4	13	1	31
pT3	15	2	11	0	28
pT4	10	2	13	0	25
total	41	10	39	1	91

Degree of differentiation: $Grade_1$, 1
$Grade_2$, 71
$Grade_3$, 12
$Grade_x$, 7

Extracapsular extension: 34[a]

Tumor emboli in lymph node vessels: 7[a]

[a] pN+ patient only, n = 50 .

(range, 0.1-9.3 years), and those alive at the last follow up examination was 5.8 years (range, 5-9.3 years).

Biochemical analysis of stefins

For biochemical analysis of Stef A and Stef B, tissue specimens from the primary tumor and adjacent normal mucosa (matched pairs) were collected during surgery. The tissue cytosols were prepared as described in details elsewhere.[6] For quantitative analysis of Stefs A and B in tissue cytosols, commercially available ELISAs (KRKA d.d., Novo mesto, Slovenia) were used, as developed at Jožef Stefan Institute, Ljubljana, Slovenia.[6] The concentrations of Stefs in tissue cytosols were expressed as ng/mg of total protein (ng/mgp).

Statistical analysis

The differences between the median concentrations of each of the Stefs in match pairs and various prognostic groups were determined using nonparametric Wilcoxon signed rank test and Mann-Whitney U-test. In the analysis of the disease-free survival (DFS, local and/or regional recurrence and/or systemic dissemination considered as event) and the disease-specific survival (DSS, deaths from disease-unrelated causes censored), Kaplan-Meier product-limit method[7] was used, and the differences between the groups were tested by the log-rank test.[8] The patients were grouped according to the cut-off concentrations of Stef A and Stef B, at which maximal differences in the survival rates were determined. All tests were two-sided and the results were considered statistically significant at the probability level of 0.05.

Results

The distribution of Stefs A and B concentrations in matched pairs is represented in Figure 1. Stef A concentrations were significantly higher in tumors compared to normal mucosa (467 *vs.* 346 ng/mgp, $P = 0.05$); however, the difference in Stef B concentrations was not significant (285 *vs.* 269 ng/mgp, $P > 0.05$). At presentation, 34 patients were staged as node-negative (clinical stage, cN_0) and 57 as node positive (cN_+). When a subgroup with clinically positive neck nodes was taken into consideration, a significant difference in Stef A (536 *vs.* 380 ng/mgp, $P = 0.03$) and Stef B (382 *vs.* 240 ng/mgp, $P = 0.02$) concentrations was observed between those with negative and those with positive necks, as determined by histopathological examination after surgery (Figure 2). In node-negative subgroup, however, this difference didn't reach the level of statistical significance. No statistically important relationship was observed between

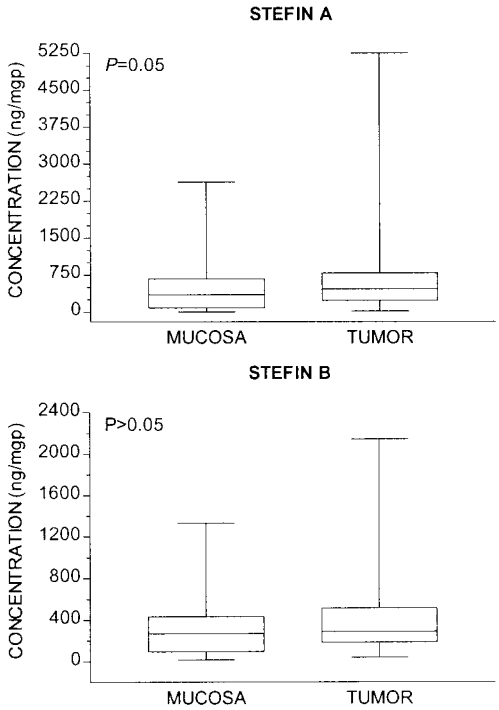

Figure 1. Concentrations of stefins in tumor and normal mucosa (n = 91). The top and the bottom of the box represent the 25th and 75th percentiles, respectively, and the ends of the bars represent the rang. The line in the box is the median value.

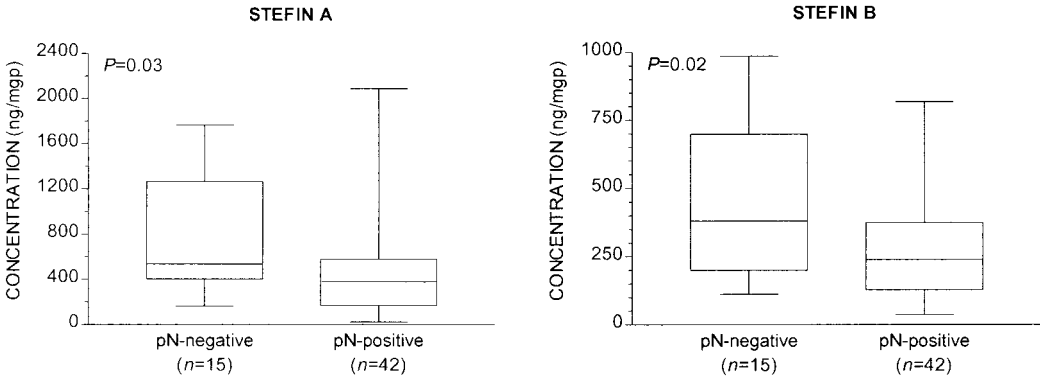

Figure 2. Distribution of tumor concentrations of stefins between patients with histopathologically determined negative and positive necks, as measured in a group with clinically palpable nodes at presentation. The top and the bottom of the box represent the 25th and 75th percentiles, respectively, and the ends of the bars represent the rang. The line in the box is the median value. n, number of samples.

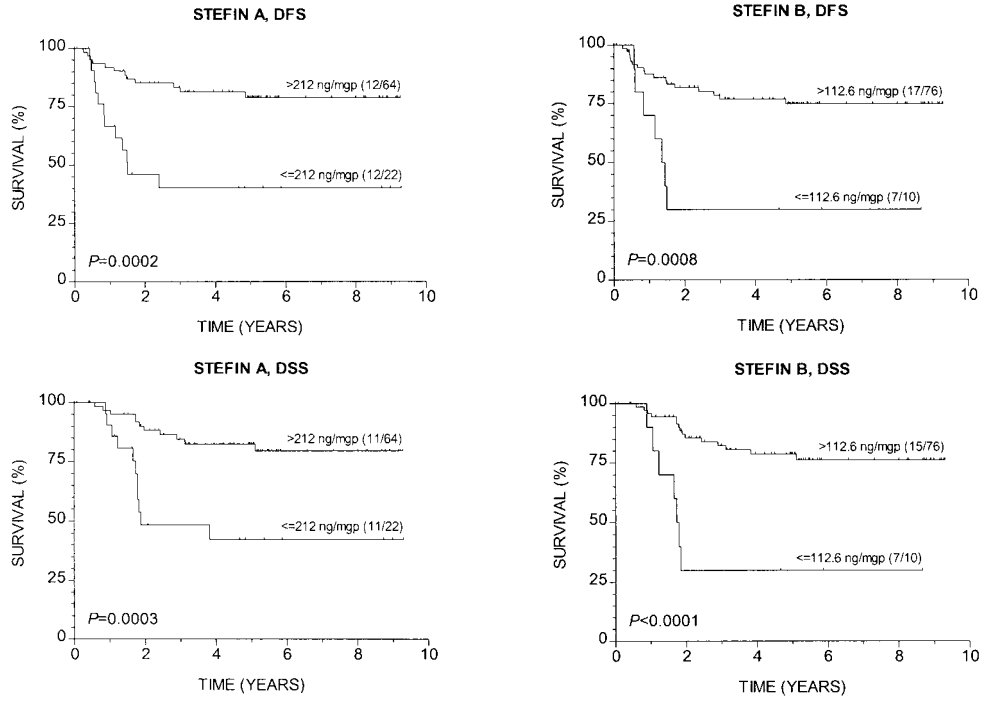

Figure 3. Disease-free survival (DFS) and disease-specific survival (DSS) of patients with respect to the optimal cut-off concentrations of stefin A and stefin B. The numbers in parentheses indicate the number of events/total in each group.

Stef concentrations and other established prognostic factors.

On univariate analysis, longer DFS and DSS correlated with higher concentrations of Stef A (P_{DFS} = 0.0002, P_{DSS} = 0.0003) and Stef B (P_{DFS} = 0.0008, P_{DSS} < 0.0001) (Figure 3). In addition, the pN_0- and lower overall stage of the disease, the absence of extracapsular extension and tumor emboli in lymph node vessels were also harbingers of favourable outcome

Table 2. Univariate and multivariate analysis of survival ($n = 86$)

Variable		Disease-free survival				Disease-specific survival			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	n	%, 5-yr	*P*-value	*P*-value	RR	%, 5-yr	*P*-value	*P*-value	RR
Stefin A									
≤ 212 ng/mgp	22	40	0.0002	0.02	0.31	42	0.0003	0.04	0.35
> 212 ng/mgp	64	79				83			
Stefin B									
≤ 112.6 ng/mgp	10	30	0.0008	NS		30	<0.0001	0.06	0.33
> 112.6 ng/mgp	76	75				79			
Age									
≤58 yrs	43	73	NS	–		75	NS	–	
> 58 yrs	43	65				68			
pT-stage									
T_{1-3}	62	73	NS	–		79	NS	–	
T_4	24	60				66			
pN-stage									
N_0	37	80	0.08	NS		88	0.01	NS	
N_{1-3}	49	61				60			
pTNM									
$Stage_{I-III}$	36	85	0.01	NS		91	0.001	0.07	3.84
$Stage_{IV}$	50	57				59			
Tumor site									
Larynx	38	77	NS	–		80	NS	–	
Non-larynx[a]	48	64				66			
Tumor differentiation									
$Grade_{1-2}$	70	70	NS	–		74	NS	–	
$Grade_3$	12	73				73			
Extracapsular spread									
Negative	52	81	0.003	0.05	4.71	87	0.0003	0.03	6.13
Positive	34	50				47			
Tumor emboli									
Negative	76	71	0.03	NS		75	0.03	NS	
Positive	7	50				33			

[a]Oral cavity, oropharynx and hypopharynx.

n, Number of patients; RR, Risk ratio; NS, Not significant.

(Table 2). Radiation dose and other classical prognostic factors didn't come out as prognostically important. In Cox multivariate regression analysis for DFS and DSS, only Stef A tumor concentrations ($P_{DFS} = 0.02$, $P_{DSS} = 0.04$) and extracapsular extension were retained in the final model (Table 2).

Discussion

In the present study we showed that Stef A and Stef B concentrations could be useful markers when deciding on treatment intensity, and reliable prognosticators in patients with SCCHN. With the analysis restricted exclusively to the patients with operable disease and

the maturity of follow-up data, places a higher emphasis on the reliability of present results.

As in our previous study on more heterogeneous group of patients,[2] only Stef A, but not Stef B, concentrations differed between tumor and normal mucosa. However, in numerous other reports on their levels and/or activity in malignant tissue compared with normal tissue the results are inconclusive, too.[1] It seems that a significant elevation of inhibitor production is not the only option when proteolytic activity in tumor tissue is increased: it is more likely that the ratio between active/non-active or functioning/malfunctioning inhibitor molecules determines the net proteolytic potential in the cells.

The new and the most important finding in the present study is that, in patients with clinically positive neck nodes at presentation (cN_+), Stef A and Stef B concentrations emerged as reliable predictors of lymph-node involvement with tumor cells. From clinical point of view, this differentiation is of utmost importance because, in a considerable proportion of patients with SCCHN (in the present series, 26 %), the nodes are enlarged due to inflammation rather than tumor infiltration. Those patients could be spared of more aggressive therapy, i.e. extensive neck surgery and/or high-dose radiotherapy, and treatment related side-effects. In this context, even though there is an overlap of individual values of inhibitor concentrations between those with pathologically positive and negative necks, Stefs alone or in combination with other biological or clinical markers that would increase their diagnostic accuracy warrant further evaluation.

In patients with clinically undetectable nodes at diagnosis (cN_0), Stefs had no potential to predict pN-stage of the disease, which is not of critical importance from clinical perspective. In this group, 76% of patients were without tumor cells at histopathological examination after neck dissection. If surgery is technically correctly performed, postoperative radiotherapy is not indicated and its side effects could be avoided. Only a minority of patients (24 %) were found to have a microscopic tumor deposits in the neck nodes which are highly curable with a moderate-dose radiotherapy, i.e. ≥ 95 % cure rate with 50 Gy.[9] As the pattern of spread of neoplastic cells from the primary tumor to regional lymph nodes is predictable,[10] the risk of geographic miss could be neglected. In addition, those neck regions with the highest risk for bearing micrometastases are usually in immediate vicinity of the primary. The most welcome consequence is that when there is an indication for primary to be irradiated postoperatively, the majority if not all nodal basins at risk are also included in the high-dose irradiation volume. Following these propositions, neck surgery is not always necessary prior to irradiation and these patients can be spared of its morbidity.

The results of univariate analysis of the survival showed that the patients with Stef A or Stef B concentrations above the calculated cut-off concentrations do significantly better than those with lower concentrations of either inhibitor. In addition, Stef A tumor concentration turned to be independent prognosticator for the risk of relapse and death in our group of patients. These results are consistent with our earlier observation[2] and further support the concept of protective role of Stefs A and B, previously raised in the studies on carcinoma of the breast[11] and lung.[12] The studies that contradict this assumption are those by Kuopio et al.[13] on breast cancer and by Kos et al.[14] on colorectal cancer. However, in the first, the Stefs' content was determined immunohistochemically, while in the other, their extracellular, i.e. serum concentrations were measured, which may reflect the involvement of Stefs in mechanisms other than the control of extracellular matrix degradation and invasion of tumor cells. Because the origin of inhibitor molecules in different sample types and/or modes of quantification of their content diffe-

red substantially from the type of analysis used in our and related reports,[11,12] a simple comparison would be inadmissible. According to our experience, the prognostic potential of immunohistochemically determined Stef expression and their serum concentrations in SCCHN is yet to be defined.[3,15]

On the basis of the presented results our conclusions would be as follows: (1) With the capability to differentiate between pathologically positive and negative necks in patients originally presented as node-positive, Stef A and Stef B could be useful markers when deciding on the extent of neck surgery; (2) Stefs A and B proved to be reliable prognosticators for the survival of patients with operable SCCHN.

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Radiol Oncol 2002; 36(2): 153.

1st Workshop on Experimental Tumour Biology

March 16 – 18, 2000
Bovec, Slovenia

Organised by

Tamara Lah (National Institute of Biology) and **Janko Kos** (Jožef Stefan Institute & Krka, d.d., Ljubljana)

Application of modern experimental methods in cancer biology generates new knowledge to understand the mechanisms of development and progression of malignant disease. With the rapid development of molecular biology, new tools are becoming available to study tumour progression – from carcinogenesis to tumour cell invasion and metastasis. The aim of the 1st Workshop in Experimental Tumour Biology was to select some topics, covered by the scientists from some research laboratories in Slovenia, working in the field of tumour biology and by their collaborators from abroad. It has become essential, especially in a small scientific community, such as Slovenia, that the researches in related areas are well and promptly informed with the latest data of their work. The exchange of information in this field is especially important for young investigators, starting their careers, and this Workshop is giving them the opportunity to get a new knowledge and to discuss the research and experimental problems with their young colleagues and senior researches.

The workshop was divided into four major sections. Carcinogenesis starts from the initial DNA damage, which is caused by external agents, present in food and beverages, by radiation and other environmental pollutants. To study their effects at molecular level, several new tests were introduced in vitro at cellular level and in vivo in animal models. This is covered in the first section on Detection of genetic alterations in cancer, where mutagenicity assays, including the newly developed Comet assays, and the tumorigenicity assays in animal models were applied to test the effects of heavy metals and bacterial toxins, possibly present in drinking water.

Next, in vitro and in vivo studies of tumour progression were discussed, where recent data on breast and brain tumour cells invasion were presented. Diagnosis of malignant progression and prognosis of the disease are clearly related and can be improved by detecting new biological markers of malignancy, such as proteolytical enzymes, especially cathepsins and their endogenous inhibitors. This was covered in the section of Immunoassays in cancer biology.

The growth of malignant tumours is associated with angiogenesis, new vessels formation, induced by the tumour cells, and understanding of the process is essential for prognosis as well as for the development of new therapeutic anti-angiogenesis agents, as discussed the in last section on Tumour angiogenesis.

We strongly believe that the success of the first workshop will lead to next meetings of this kind and we hope that they will become traditional in the future.

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Genetic toxicology: from exposure detection to cancer prevention

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There is a general concern that many environmental chemicals to which humans are exposed are genotoxic and may cause cancer. The main problem is not only to identify the environmental genotoxic pollutants, but also to characterise their mode of action at cellular and molecular levels. To identify genotoxic environmental pollutants we are using selected in vitro short-term assays.

For fast screening for the presence of genotoxic substances in wastewater and fresh waters samples we use modifed SOS/*umu* test and Salmonella/microsomal assay. The modified assays are sensitive enough to detect low concentrations of genotoxic pollutants in non-concentrated water samples. We have demonstrated by using bacterial strains with elevated nitroreductase and/or O-acetyltransferase activity, that nitro polyaromatic hydrocarbons are important genotoxic contaminantns in water. We suggest to use the modifed SOS/*umu* for rutine controling the efficiency of wastewater treatment plants.

For detection of mutations, which are the consequence of DNA damage and the processing of the damaged DNA, we are using the Comet assay or single-cell gel electrophoresis (SCGE). This is a sensitive method for detecting DNA strand breaks at the level of individual cells. Cells embedded in agarose are lysed, subjected to alkaline unvinding, electrophoresed and stained. DNA, broken and relaxed in the electric field, migrates toward the anode, resembling a shape of a comet with bright fluorescent head and a tail region, which increases, as the DNA damage gets more severe. The "comets" are measured and analysed using video image analysis: DNA damage is quantified by tail length, tail moment and percentage of DNA in the tail. In our hands, the comet assay proved to be a valuable tool to study the biochemical and physicochemical mechanisms of DNA damage and repair. Based on the Comet assay we are developing a biomarker for detection differences in DNA repair capacity in populations exposed to heavy metals (Cd and Pb) compared to non-exposed. As heavy metals inhibit DNA repair, we supose that in populations exposed to heavy metals cellular DNA repair capacity is affected, leading to enhanced risk for cancer.

For identification of cancer preventive agents and potential antimutagens, we found, that the extract of *Salvia officinalis* inhibited UV induced SOS response and mutagenesis in bacteria. The results indicate that *Salvia officinalis* extract acts as an inhibitor of SOS functions as well as a promoter of DNA excision repair processes. For screening large number of samples we use SOS/*umu* test, modifed for detection of potential antimutagens. The samples that show antimuatgenic potential in SOS/*umu* test are than tested for antimutagenic activity in bacterial reverse mutation assay using different strains to allow insight in to the mode of action. Using this approach, we tested 121 mushroom extracts

for antigenotoxic activity. Seven of them inhibited UV induced SOS response by more than 50 % and three of them were effective also against oxidative damage.

Radiol Oncol 2002; 36(2): 156-8.

Cadmium induced DNA damage in human hepatoma (Hep G2) and Chinese hamster ovary (CHO) cells

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Introduction

Cadmuim (Cd) is one of the most important heavy metal environmental toxicants. It accumulates in human tissues, particularly in kidney and liver. Cd is classified as probable human carcinogen by IARC.[1] The genotoxic potential of Cd is rather weak and restricted to high cytotoxic concentrations. However, at low concentrations Cd enhances genotoxicity of other DNA damaging agents.[2] It was shown that Cd interferes with nucleotide excision repair (NER) by inhibiting DNA damage recognition and incision step of NER.[3] The aim of the study was explore the DNA damaging potential of Cd and its interference with the repair of UV induced DNA damage *in vitro*, using Comet assay. We studied the induction of DNA single strand breaks (ssb) by low nanomolar concentrations of $CdCl_2$ after different duration of exposure on human hepatoma cell line (Hep G2). The effect of $CdCl_2$ on the repair of UV induced DNA damage was studied in Chinese hamster ovary cells (CHO). The results of the formation and disappearance of DNA ssb after different periods of recovery after the UV irradiation, reflecting the nucleotide excision repair (NER) kinetics, are presented.

Materials and methods

Cell lines

Human hepatoma cells (HepG2) cells were cultured in Williams Medium containing 10 % FBS at 37 °C in humidified atmosphere with 5 % CO_2. Chinese hamster ovarian (CHO) cells were cultured in F-12 medium containing 10 % FBS at 37°C in humidified atmosphere with 5 % CO_2.

Treatment of cells

HepG2 cells were incubated with low, non-toxic concentrations of CdCl2 (10 nM, 100 nM and 1000 nM) in complete growth medium for 3, 6, 9, 12, 24 and 72 h. After the incubation period the cells were harvested and subjected to alkaline single cell electrophoresis (Comet assay). CHO cells were seeded in 25 cm^2 tissue culture flasks one day prior to treatment with Cd. Five hours prior to the UV irradiation the cells were treated with 1 μM and 10 μM $CdCl_2$ in complete growth medium. After the treatment the cells were washed with PBS, harvested, suspended in PBS and UV irradiated (20'', 50-cm distance). The irradiated cells were subjected to Comet assay after the recovery period of 0, 10, 20, 30 and 60 minutes at 37 °C.

Comet assay

The cells (HepG2 or CHO) were embedded in 1 % LMP agarose on pre-coated microscope slide and lysed at 4 °C for 1 h (2.5M NaCl, 100mM EDTA, 10 mM Tris, 1 % Triton X-100, pH10). The slides were then placed in the electrophoresis solution (300 mM NaOH, 1 mM $EDTANa_2$, pH13) for 20 min at 4 °C to

allow DNA unwinding and electrophoresed for 20 min at 25V (300 mA). After electrophoresis slides were neutralized (0,4 M Tris, pH 7,5) and stained with EtBr. 50-100 cell nuclei per each experimental point were examined at 400 magnification using a fluorescence microscope (Olympus) and analyzed with the software VisCOMET. The DNA damage is expressed as comet tail moment, which is defined as product of the comet length and percentage of DNA in tail.

Results and discussion

Figure 1 shows the effects of low non-toxic concentrations of $CdCl_2$ on DNA damage in HepG2 cells. Level of DNA damage was assessed after different periods of incubation with $CdCl_2$ by measuring the increase of comet tail moments. Incubation with 10 nM, 100 nM and 1000 nM $CdCl_2$ caused a dose-dependent increase of DNA damage. The DNA damage increased also with the increasing time of exposure to $CdCl_2$ up to 12 h. However, when the cells were incubated for 24 h the DNA damage was lower than after 12 h incubation. After 72 h incubation DNA damage was detected only in cells treated with 1000 nM $CdCl_2$. This time dependent decrease of DNA damage can be due to the Cd mediated induction of the synthesis of metallothioneins, which are known to play role in cellular defence mechanism against Cd toxicity.[4]

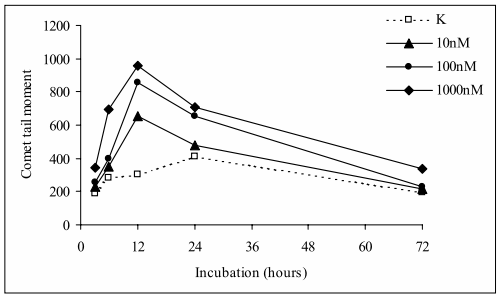

Figure 1. Comet tail moment of HepG2 cells incubated with CdCl2 for 3, 6, 12, 24, and 72 h. 100 comets per experimental point were analysed by image analysis system.

UV irradiation induces pyrimidine dimers and 6-4 photoproducts that are repaired predominantly by NER. With the Comet assay ssb are detected, which reflect the incision step of the NER. In $CdCl_2$ pretreated cells the tail moment was lower than in control cells (Figure 2) indicating that $CdCl_2$ prevented the incision step of NER. After 60 minutes of recovery the residual ssb were higher in $CdCl_2$ treated cells compared to the control, which might reflect either slower NER or inhibition of ligation step of NER. This result confirms the interference of Cd with NER.

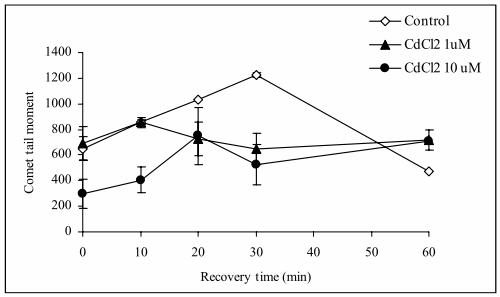

Figure 2. DNA repair kinetic of UV induced DNA damage in CHO cells. Non-treated or CdCl2 pre-treated cells were exposed to UV irradiation and than incubated at 37oC. At different intervals samples were taken for comet assay. 100 comets per experimental point were analysed by image analysis system.

Conclusion

In conclusion, our results in Hep2G cells showed that ssb were induced at 10 nM of $CdCl_2$, which is the concentration that corresponds to the concentrations of Cd found in blood of environmentally exposed population.[5] This damage was detected only after short time of exposure. In CHO cells, we demonstrated, that when cells are exposed to $CdCl_2$ for a short time, the repair of UV induced DNA damage was inhibited. Further experiments are in progress to explore the role of metallothioneins in protection against genotoxic and cogenotoxic effects of Cd.

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In vitro genotoxicity of microcystin-RR on primary cultured rat hepatocites and Hep G2 cell line detected by Comet assay

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Introduction

Microcystins are hepatotoxic cyclic heptapeptides produced by different species of bloom forming cyanobacteria (*Microcystis, Anabaena, Nostoc Oscillatoria*).[1] The primary target of the toxin is the liver. The uptake of microcystins into the hepatocites occurs via carrier-mediated transport system. Microcystins cause cytoskeletal damage, necrosis and pooling of blood in the liver, with a consequent increase in the liver weight. The cause of death is a massive hepatic haemorrhage.[2]

Microcystins are inhibitors of serine/threonine protein phosphatases 1 and 2A and act as tumor-promoters.[3] Ito and coworkers[4] demonstrated that microcystins induced neoplastic nodules in the liver after repeated injections without an initiator, which indicates that they might act also as tumor initiators.

The aim of our studies was to elucidate possible genotoxic effects of microcystin-RR (MCYST-RR) at molecular level using Comet assay. The Comet assay is a sensitive method for detection of DNA strand breaks at the level of a single cell.[5] DNA single-strand breaks can lead to mutations, which are the first step in carcinogensis.

Materials and methods

Primary cultured rat hepatocytes and the cell line Hep G2

Primary rat hepatocytes were isolated from rat liver by a three-step collagenase perfusion method described by Doyle.[6] The isolated hepatocytes and Hep G2 (human hepatoma cell line) were cultured in Williams Medium containing 10 % FBS for four h at 37 °C in humidified atmosphere with 5 % CO_2.

Cell treatment

The primary cultured rat hepatocytes were rinsed with phosphate buffer saline (PBS) (Mg^{2+} and Ca^{2+} free) and then incubated with different concentrations (0.01, 0.1 and 1 μg/ml) of microcystin-RR (MCYST-RR) in Williams Medium containing 10 % FBS for 3 and 13 h. Control cultures were treated with solvent (methanol) only. After the treatment hepatocytes were rinsed with PBS, trypsinised and centrifuged at 1000 rpm for 10 min. Similarly, the Hep G2 cells were incubated with different concentrations of MCYST-RR in Williams Medium containing 10 % FBS for 13 h, rinsed with PBS, trypsinised and centrifuged at 1000 rpm for 10 min

Comet assay

30 μl of a freshly prepared suspension of cells ($3{,}5x10^5$) and 70 μl low melting point agarose were added to a fully frosted microscope slide,

precoated with 80 µl of 1 % normal melting point agarose, covered with the top slide. The top slide was removed after solidifying and the cells were lysed at 4 °C for 1 h (2.5M NaCl, 100mM EDTA, 10 mM Tris, 1 % Triton X-100, pH10). The slides were then placed in the electrophoresis solution (300mM NaOH, 1mM EDTANa_2, pH13) for about 20 min at 4 °C to allow DNA unwinding before electrophoresis. Samples were electrophoresed for 20 min at 25 V (300 mA). After electrophoresis the slides were placed in 0.4 M Tris buffer (pH7.5) for 15 minutes in order to neutralise and then stained with ethidium bromide (5 µg/ml). The 100 cell nuclei were examined at 400 magnification using a fluorescence microscope (Olympus) and analyzed with the software VisCOMET. MTT cytotoxicity test was performed as described previously by Immamura.[7]

Results

Significant increase in DNA damage of the primary cultured rat hepatocytes treated with MCYST-RR when compared to the (solvent) control was observed only after prolonged treatment for 13 h. The MTT test showed that MCYST-RR was not toxic at the tested concentrations (results not shown).

Hep G2 cells were treated with the same concentrations of MC-RR and for the same time as primary cultured rat hepatocytes. The results showed a dose-response similar to that of isolated cells. MC-RR was not toxic to Hep G2 cells at the tested concentrations.

Discussion

The genotoxic potential of microcystin RR was evaluated since it is the most frequent and abundant hepatotoxin in Slovene surface water bodies.[8] The results demonstrated that MCYST-RR induced dose dependent DNA damage in both, primary cultured rat hepatocytes and in Hep G2 cell line at the nontoxic concentrations. With the comet assay DNA damage has already been demonstrated in primary cultured rat hepatocytes after the

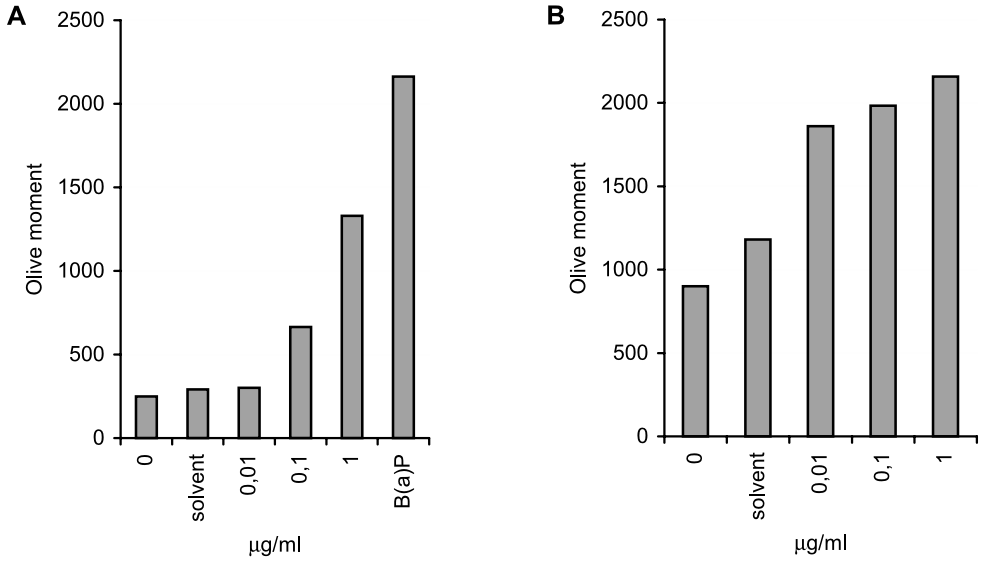

Figure 1. Olive tail moment of primary cultured rat hepatocytes (A) and the cell line Hep G2 (B) treated with different concentrations of microcystin-RR. The cells were treated for 13 h. Mean values are presented. Methanol (solvent) was used as negative and B(a)P was used as a positive control.

treatment with MCYST-LR (1 μg/ml).[9] In their experiments the DNA damage was induced after 4 h treatment, while in our experiment MCYST-RR was effective only after treatment for 13 h. MCYST-RR is known to be 10 times less biologically active in comparison to MCYST-LR.[10] This could explain the difference in exposure time, needed to induce DNA damage. The fact, that the effects are time dependent corroborates the importance of chronic *in vivo* experiments for the extrapolation of health risks.

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Co-operative effects in tumorigenicity. The microcystin example.

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Introduction

Cyanobacteria have been implicated in many deaths of livestock, wildlife and human throughout the world.[1] They produce a broad range of biologically active substances including proteinase inhibitors[2], endotoxins (LPS), which are generally present in gram-negative bacteria[3], and a variety of other toxic compounds.[4] These substances are released in the water environment during the senescence of the bloom and can penetrate in the water supply system. Little attention has been paid to possible synergistic interactions between these biologically active substances in tumor promotion and tumor initiation.

With few exceptions[5], the vast majority of experiments used for the human risk assessment of cyanobacteria have been performed using purified microcystins.[6] To evaluate liver injuries such as cirrhosis and hepatocellular carcinoma high doses of pure microcystins have been used.[7] The present paper presents an attempt to verify the possible synergistic effects of different biologically active substances we have tested the toxic effects of lyophilized hepatotoxic cyanobacteria in comparison to the effects produced by the same amounts of purified microcystins.

Materials and methods

The toxicity was estimated by a mouse bioassay and the microcystins isolated and determined as described elsewhere.[8] Acute and sub chronic experiments were performed on Wistar rats and New Zealand rabbits, as described previously.[9] The toxic material was injected intraperitoneallly (*i.p.*).

Results and discussion

In our experiments we have been able to detect precancerosis after application of much lower amount of microcystins in lyophilized cyanobacteria (2 LD_{50} injected during the 5 weeks period). This amount is on average 4 fold lower, than the amount used by other authors in order to produce similar injuries by *i.p.* application of purified microcystins. Since endotoxins (LPS) present in cyanobacteria have a profound effect on detoxification of microcystins by the suppression of glutathione S- transferase activity[10] they may enable the accumulation of microcystins in the liver of exposed organisms (Figure 1).

The persistence of microcystins in liver cells may be responsible for the more pronounced changes in the liver of animals treated with whole cyanobacteria in comparison to the animals treated with pure microcystins (Figure 2).

Experimental data imply that microcystins can no longer be treated merely as tumor promoters since it has been demonstrated that prolonged exposure to microcystins can induce neoplastic nodular formation.[7] Additionally, DNA damages have been observed as a result of exposure to microcystins.[11]

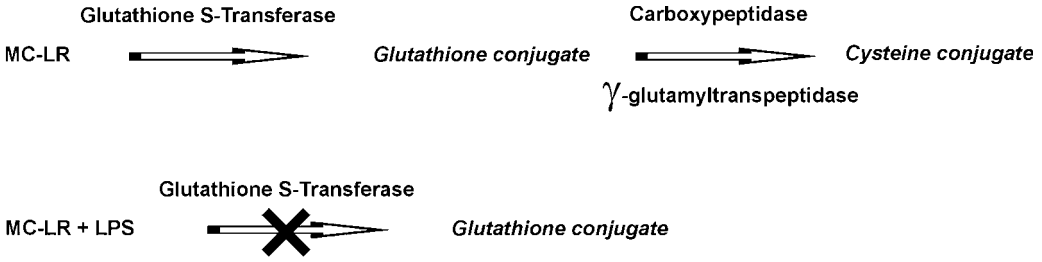

Figure 1. Inhibition of microcystin detoxification in the presence of endotoxin (LPS) as suggested by Pflugmaher and coworkers.

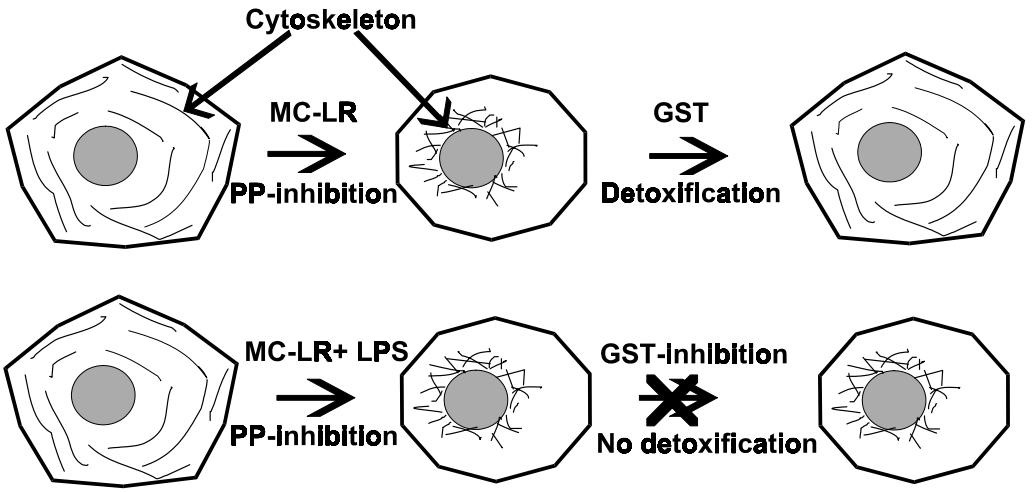

Figure 2. Prolonged action of microcystis on liver cells due to the inhibition of detoxification.

We must be aware that several other toxic or carcinogenic substances found in environmental waters can be dissolved, adsorbed or trapped on the surface of cyanobacteria. Therefore, in our opinion the health risk of cyanobacteria can not be established only on the basis of microcystin contents, meaning that the risk is underestimated.

Conclusion

New experimental data on cyanobacterial toxicity suggest that the extrapolation of the health risk of microcystins from acute and sub chronic experiments performed mainly with purified toxins is not adequate. Other biologically active substances normally present in cyanobacterial blooms can amplify their toxic effects. There are also evidences, that microcystins are able to damage the genetic material of exposed animals and can even induce formation of neoplastic nodular formation. Therefore, at least in our opinion, the health risk of natural cyanobacterial blooms is underestimated.

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Chronic exposure to cyanobacterial lyophilisate reveals stronger effects than exposure to purified microcystins – a MRI study

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Introduction

Microcystins are potent hepatotoxins, tumor promoters, and carcinogens. Although they are present in surface water bodies worldwide little has been done to assess the effects of chronic exposure to these substances in human population. This may partly be due to the fact that acute intoxication by these substances is rare in man. Recent experience from Brazil where a number of dialysis patients died due to the presence of microcystins in the water used for dialysis is a serious warning. Chronic exposure to microcystins is less dramatic, but more widespread. Therefore the aim of our study was to test magnetic resonance imaging (MRI) as a noninvasive and harmless method for the early detection of changes in liver tissue after chronic exposure to microcystins. It should be emphasized that living organism are in most cases chronically exposed to toxic cyanobacteria and/or to their dissolved contents, which consist of a broad range of biologically active substances and not only microcystins.[1-4] Therefore the second aim of our study was to assess the possible synergistic interactions between these substances in chronic intoxication by using either purified microcystins or cyanobacterial lyophilisate (CL).

Methods

Male albino rats (Wistar) weighing 165-190 g were used in all experiments. The animals were kept at standard room condition (room temperature 24 °C, daily-night interval at 12 hours, exposed to 60 lux artificial light). The number of animals was kept at minimum. The experiments were performed with the guidelines of IST. Either cyanobacterial lyophilisate or purified microcystins were injected i.p. in the intervals of 3 days for 2 months, the total cumulative dose of microcystins (either in the lyophilisate or purified substance) was 2 LD_{50}.

Magnetic resonance imaging of the rat's liver was performed on a Bruker Biospec system with a 2.35 T horizontal bore magnet on animals anesthetized with i.p. injection of Xylazin (15 mg/kg), Ketanest (100 mg/kg) and atropine (0.3 mg/kg). In this study, standard T1 weighted spin-echo magnetic resonance imaging method with echo time 18 ms and repetition rate 400 ms was used. Signal averaging of 10 signal acquisitions was used to improve the signal to noise ratio. In order to extract the liver volume, rats were imaged in seven consecutive slices in two experiments, first in transverse and then in coronal slice orientation. Imaging filed of view and slice thickness was at each experiment adjusted so that imaging volume covered the whole liver region. Thus, field of view was in the range 8-10 cm and slice thickens 3-4 mm. T1 weig-

hted MR images had good contrast between liver and surrounding tissues that allowed precise liver region selection and its area calculation for each slice. Liver volume was later calculated as a sum of liver areas in all slices multiplied by the slice thickness.

Results and discussion

In acute experiments MRI revealed enlargement of liver in both groups of experimental animals, and there was no difference whether they received only purified microcystins or the same amount of microcystins present in the CL. Significant differences have been observed between the group that received CL or purified microcystins only in the chronic experiments. Liver from the animals injected with the microcystin- LR showed only minor changes on the signal intensity on MRI images, and patho -morphological examination of abdominal organs on sacrificed animals showed degenerative changes in one animal only. The MR images of the liver from all animals injected with CL showed irregular changes in the signal intensity and nodular formations have been observed. Patho -morphological examination of sacrificed animals showed granular structure of liver edges, and enlargement of kidneys. However, degenerative changes characterised as periportal fibrosis and fatty infiltrations were observed in both groups of chronically treated animals. Changes were more pronounced in the case of the animals treated with CL. No tumours were detected in any of the treated animals, which is in agreement with the data from other authors[5] that showed that longer exposures and higher dosages are necessary to produce carcinogenic effects.

Extracts from toxic cyanobacteria induce DNA damage in vitro as well as in vivo.[7] This implies that microcystins can no longer be treated merely as tumour promoters. The enlargement of kidney shown in our experiments suggests that exposure to toxic cyanobacteria also affects other organs, which has already been proposed.[8] Microcystins could be responsible for these effects, but the combined action of the constituents of cyanobacteria is evidently important as kidney enlargement has been observed only in the group of animals treated with CL. The data show that not only microcystins but also other components of the cyanobacterial bloom can affect the health of population.[6] Therefore the health risk of hepatotoxic cyanobacteria estimated from the microcystin content is underestimated.

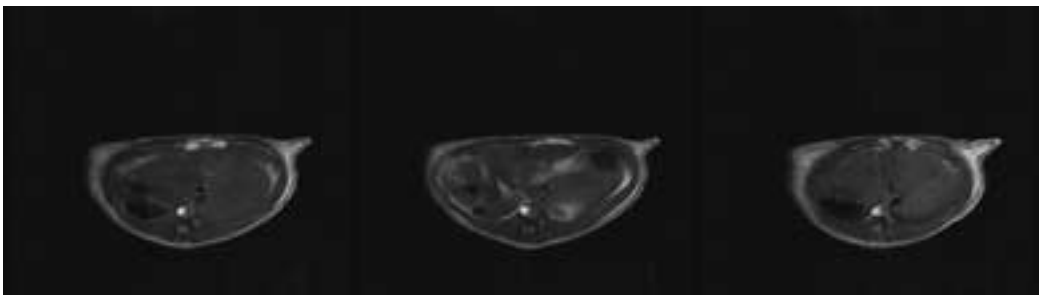

Figure 1. MR images of the liver exposed to cyanobacterial lyophilisate or to purified microcystins alone. A. Control liver is of a dark gray and homogeneous colour. B: Chronic exposure to cyanobacterial lyophilisate (CL) results in liver degeneration shown as non-homogeneous signal from the liver seen as white patches. C) Chronic exposure to microcystins gives rise to only slight changes in the liver structure as seen by MRI.

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Tumor progression and invasion

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Cancer is the disease of the gene. The very first event that damages nuclear DNA is initiated by an initiator (substance, radiation), which may cause different effects, such as mutations, DNA breaks, etc, which may or may not cause cancer. Additional hits, which do lead to the development of cancer, are caused by tumor promoters. At present, detailed knowledge of all the events leading to a malignant disease, are not yet understood. Molecular description of tumor progression envisages that each type of cancer will progress in stages: the step-wise genetic changes accompanying this progression, are unique to each type of cancer. To study these events, various in vitro and in vivo approaches were developed. The first part of the workshop will be dedicated to the methodology used to identify DNA damage, while the second part will be dedicated to the experimental models used to identify biological markers, associated with tumor and/or endothelial cell invasion.

Metastasis of primary tumors is comprised of biologically distinct steps and is rather inefficient process. Local tumor cell invasion is a common denominator in many of these steps and was first described by Liotta[1] as a three-step process, where, first specific attachment (I) is followed by the induction and/or release or hydrolytic – proteolytic – enzymes, which degrade (II) extracellular matrix components, thereby facilitating tumor cell locomotion (III) and penetratioan into the host tissues. Several experimental models are used to study the molecular mechanisms of invasion. Most in vitro invasion assays are using natural tissues, such as amnion membrane, eye lens and fragments of various tissues, such as chicken heart, mouse liver, lung, etc. Organotypic co-culture models were mostly used for studying brain tumor invasion. The most simple are invasion assays using modified Boyden chambers[2,3] and Matrigel or other isolated proteins of extracellular matrix. However, the results may not be reproducible even when using the same system, due to several methodological problems, comprising variability in composition, concentration and preparation of Matrigel (or other proteins), quality of filters, treatment of cells (pH, trypsinization, organic solvents), chemoatractants, time of the assays and methods used for the quantitation of the invasion. The data in the literature confirmed that various proteinase inhibitors were effective in partial inhibition of invasion, indicating that one or several proteinases are involved in this process. The results on inhibition of invasion of breast tumour cells, using synthetic and natural (peptide) cysteine proteinase inhibitors are not yet conclusive, due to variability of methodological approaches, described in the literature.

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In vitro invasion of transfected human breast epithelial cells MCF10A-neoT

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Introduction

Tumor invasion and metastasis are responsible for the progression of malignant disease. These processes are facilitated by the upregulation of various types of proteinases. In recent years, experimental and clinical studies have been carried out using inhibitors of MMPs,[1,2] plasmin and plasminogen activators,[3] as well as cysteine proteinases.[4,5,6,7] They inhibit either growth,[7] motility[8] or the invasive potential[4,5] of various types of tumours. The aim of the study was to evaluate the effectiveness of synthetic and peptide proteinase inhibitors in reducing *in vitro* invasion of MCF10A cells, transfected with ras oncogene.

Material and methods

MCF10A-neoT cell line was established by transfection of MCF10A cells with T24 c-Ha-ras oncogene[9] and has an acquired ability to grow as xenograft in nude mice, forming small nodules, which progress sporadically into invasive carcinomas of different histological types. Cells were grown to 80 % confluency and 24 h before harvesting, the medium was replaced with SFM (serum free medium).

Invasion assay

The method described by Albini and co-workers[10] was used with minor modifications. Polycarbonate filters (Costar, USA) with 12 μm porosity were coated with fibronectin (25 ng/mm, Sigma, Germany) on the lower surface and with Matrigel (0.9 μg/mm, Becton Dickinson, USA) on the upper surface, dried overnight and reconstituted with 200 μl SFM for 1 h at 37 °C. Maximum inhibition of invasion was observed using SFM containing proteinase inhibitors (Bachem, Switzerland) at the following non-cytotoxic concentrations: 10 μM E64, 100 μM E64-d, 20 μM Ca-074, 0.5 μM Ca-074Me, 20 μM Z-FA-FMK, 0.5 μM Clik 148, 50 μM Z-FF-CHN_2, 1 μM pepstatin A, 100 μg/ml aprotinin and 10 μM BB94. Cytotoxicity was determined using MTT (1-(4,5 dimethyltiazol-2-yl)-2,5 diphenyl tetrazolium bromide) viability assay. Cells were harvested and seeded (200,000 cells in 0.5 ml SFM) to the upper chamber. After 21 h incubation, MTT (Sigma, Germany) at the final concentration of 0.5 mg/ml was added.[3] The cells were further incubated for 3 h at 37 °C to allow the formation of formazan crystals. The crystals from upper and lower chambers were separated and pelleted by centrifugation at 15000 rpm for 5 min and dissolved in 1 ml of DMSO. Optical density (OD) was measured at 570 nm (reference filter 690 nm). Invasiveness of the cells was calculated as the ratio of the OD in the lower chamber to the sum of ODs in the lower and upper chambers.

Results

The ability of inhibitors of cysteine, aspartic, serine and metalloproteinases to reduce inva-

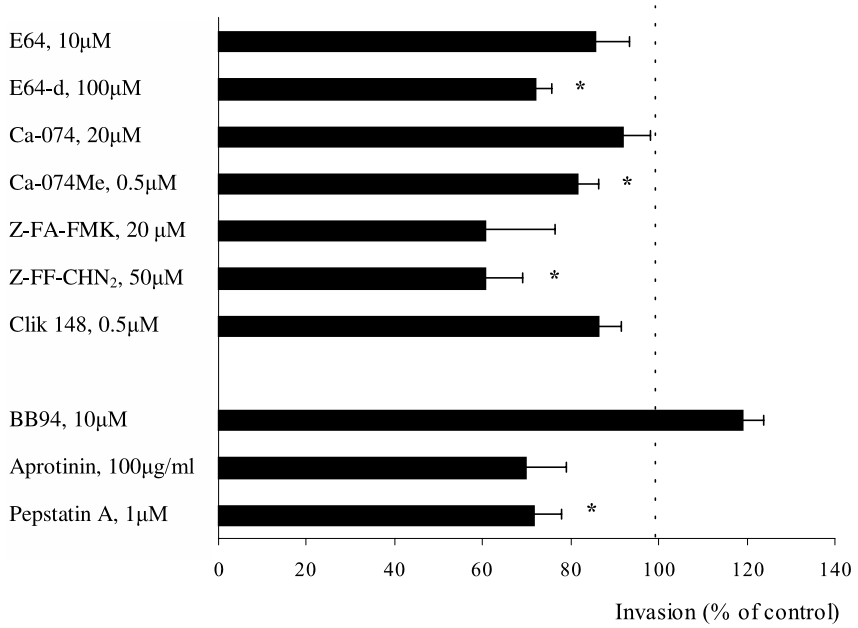

Figure 1. Inhibition of MCF10A-neoT cells by synthetic inhibitors of invasion.

sion of MCF10A-neoT cells was determined (Figure 1). Membrane-permeant cysteine proteinase inhibitors, E64-d (28 %) and Z-FF-CHN_2 (40 %), were the most effective, followed by the selective CatB inhibitor, Ca-074Me (about 20 %). Inhibition of invasion by Z-FA-FMK (40 %) and the selective CatL inhibitor, Clik 148 (14 %) was not statistically significant. Among the inhibitors of other types of proteinases, only pepstatin significantly inhibited the invasion (28 %). Aprotinin reduced the invasiveness to the similar extent. The broad spectrum matrix metalloproteinase inhibitor BB94 did not inhibit invasiveness.

Data is expressed as percentage of the control. Error bars depict standard error of the mean values of three independent experiments. Statistical significance (*) was determined by two tailed t-test with assumed equal variance, and $p < 0.05$ was considered significant.

Discussion

The MCF10A-neoT cell line was used to determine the effect of proteinase inhibitors on invasion. Cysteine proteinase inhibitors were found to be more effective than the inhibitors of other classes of proteinases. Therefore, we conclude that cysteine proteinases contribute significantly to the process of invasion. CatB inhibitors proved more effective than CatL inhibitors, suggesting that CatB plays a more important role than CatL. Since the derivatives of CatB inhibitors, which can enter the cells, were found to be most effective, the cells probably degrade collagen also intracellularly, as reported previously. The aspartic proteinase inhibitor pepstatin A also reduced the invasion, so CatD may also be involved in breast tumor cell invasion.[12] Similar to reports in human esophageal and ovarian carcinoma cells *in vivo*[13], we did not observe inhibition of invasion by the broad spectrum inhibitor of MMPs, BB94. This is in contrast to the previous reports of its inhibitory effect

if invasion in other cell types.[4,5] Such discrepancies may reflect differences in the expression and activation of MMPs in various cells. Our results support the hypothesis that the cysteine proteinase CatB plays an active role in invasion of transformed human breast cell lines. These findings could have an impact on the search for new anti-invasive and anti-metastatic agents

Acknowledgements

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The effect of E-64 and monoclonal antibodies on proliferative and invasive activity of ras transformed human breast epithelial cell line MCF10A neoT tested in *in vitro* assays

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Introduction

The characteristic of malignant tumours is their potential to invade normal host tissue and to metastasize to distant organs.[1,2] Tumour cell invasion of the extracellular matrix (ECM) is described[3] as a multi-step process. First, tumour cells need to be attached to the components of the ECM. Next step is the local degradation of ECM components, followed by the migration of the tumour cell through locally modified matrix. Invasive behaviour of tumour cells requires proteolytic activity associated with the concerted action of various intra and/or extracellular proteinases, including a cysteine proteinase cathepsin B. Cathepsin B was found localized on the surface of different brain and breast cancer cells, including MCF10A neoT.[4] Another prerequisite for tumour cells to form distant metastases is tumour cell proliferation.[2]

The effect of E-64, an irreversible cysteine proteinase inhibitor and various monoclonal antibodies raised to human cathepsin B, was tested on ras transformed human breast epithelial cell line MCF10A neoT, using *in vitro* proliferation and invasion assays.

Materials and methods

Hybridoma technique, first demonstrated by Köhler and Millstein in 1975[5], was used to prepare mouse anti-cathepsin B monoclonal antibodies. Recombinant cathepsin B was used for immunization of Balb/c mice. For *in vitro* proliferation and invasion assays we used MAbs, derived from two clones of hybridoma cells.

The MCF10A neo T, ras transformed human breast epithelial cell line was used in the in vitro assays. The cells were cultured in DMEM/F12 (1:1), with 5 % foetal calf serum (FCS) and supplemented with insulin, epidermal growth factor, hydrocortisone and antibiotics at 37 °C and 5 % CO_2 in humidified atmosphere, to about 70-80 % confluency. The assays were performed using the same DMEM/F12 medium, however, FCS used was purified on the affinity chromatography column using immobilized papain.

The MTT-cell proliferation assay[6] was used to assess possible influence of inhibitor and antibodies on tumour cell proliferation. It is a quantitative colorimetric assay based on cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into water insoluble, dark-blue formazan crystals by the mitochondrial enzyme succinate-dehydrogenase.[7] Assays were performed in quadruplicates.

To study the effects of MAbs and E-64 on tumour cell invasion, Costar Transwells with 12 mm polycarbonate filters and 12 μm pore size were used. Filters were coated with Matrigel (Becton Dickinson).[8] Assays were performed in triplicates.

Results and conclusions

In the proliferation assay, MAbs derived from clone A and E-64 didn't show any effect in the concentration range tested (2-0,01 μM and 100-0,1 μM). MAbs derived from clone B expressed a slight anti-proliferative effect in a dose-dependent manner (2-0,01 μM).

Results of in vitro invasion assay show a strong inhibitory effect expressed on the invasion of MCF10A neoT cells by E-64. MAbs derived from clone B had no effect, whereas MAbs from clone A expressed a 20 % inhibition compared to control at 1 μM concentration.

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Brain tumour migration and invasion: The role of in vitro model systems

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Human primary (intrinsic) brain tumours rarely metastasis to distant organs, however they do show a marked propensity for diffuse infiltrative invasion of the contiguous, normal brain tissue. This is arguably the most important biological feature of this group of – predominantly glial – neoplasms. Single neoplastic glia may migrate several millimetres, or even centimetres from the major tumour mass and there is increasing evidence that during the migratory phase these cells transiently arrest from the cell cycle therefore rendering them refractory to therapeutic radiation. Moreover, they are protected from the action of the majority of cytotoxic drugs by virtue of their investment within areas of intact blood-brain barrier. These migratory, so-called "guerrilla" cells later return to the division phase, under hitherto unknown microenvironmental cues, to form local recurrences of the primary tumour.

The terms "migration" and "invasion" in the context of oncology frequently tend to be used interchangeably however, strictly speaking migration refers to the simple movement of cells within a tissue without causing specific damage to the host tissue while invasion infers the movement of cells with consequent detriment to the normal cellular elements. Ideally, in order to elucidate the underlying mechanisms and patterns of invasion this phenomenon should be studied in *in vivo* model systems.

Animal models of brain tumour, however, do not generally fulfil the criteria required for such studies. Indeed, while intracranial tumours induced by chemical carcinogens such as ethyl nitrosourea (ENU) do invade the brain, they occur at inconsistent locations and at differing latency (which is long) and incidence. Transplantable glial tumours tend to have rapid growth rates, growing by expansion rather than diffuse invasion and\ showing only limited dissemination, generally along the vascular basal laminae. To date the only convincing demonstration of invasion in an animal model comes from the direct xenotransplantation of human biopsy tissue into immunodeficient rats. This model, however, suffers from lack of reproducibility and therefore ineffective statistical accountability.

In order that invasion of glial tumours can be studied in the laboratory it has been necessary to develop a variety of *in vitro* approaches. Although each of these approaches suffers from intrinsic flaws they have collectively provided considerable information regarding the cellular mechanisms and molecular pathways which underlie the process of brain tumour invasiveness.

The design of such models is of great importance since the culture microsystem exerts an influence on the invasive and motile properties of neoplastic cells.

Although a simple scratch across a confluent monolayer facilitates monitoring of migration, more sophisticated techniques have been developed. For example, cells may be seeded into cloning rings on extracellular matrix protein coated substrates and incubated in culture for a 12 hour period, then the ring may be removed and migration away from the cell colony assessed by sequential photomicrography.

It is also possible to examine the movement of tumour cells across the substrate of a culture dish by time-lapse video photomicroscopy. Here, the substrate can be varied and putative chemorepellant or chemoattractant factors, as well as agents that may promote or retard invasion, may be added to the growth medium and their effects studied over a period of many hours. Using this approach the trajectories of cell movement may be tracked and the intervals between cell divisions logged.

Another method employs modified Boyden chambers. In essence, commercially available "Transwell" units, incorporating inserts with polycarbonate membrane filters (porosities of 8 to 12 microns are generally suitable for the study of human neoplastic glia), are set up with a chemoattractant containing medium (eg platelet-derived growth factor or tumour cell conditioned medium) in the lower chamber and cells for assay are seeded onto the top of the filter in the upper chamber (the two chambers are separated by the filter alone). After a pre-determined period of incubation, filters are removed and cells which have migrated to the lower side of the filter may be stained with simple haematological stains such as modified Papanicolau (Diff-Quik) or by immunocytochemical methods: electron microscopy may also be carried out. Cells on the non-migratory (upper) side of the filter are either ignored, as unfocussed cells, or may be removed by scraping, counts can then be made of migratory cell populations. By coating the polycarbonate filter with a thin layer of extracellular matrix (ECM) components such as "Matrigel" or with growth inhibited, viable non-neoplastic cells, the migration assay can be converted to an assay for invasiveness. Such assays not only monitor the motile propensity of cells but also require that the cells adhere to and subsequently degrade the ECM in order to permit invasion.

Three-dimensional confrontational models where normal tissue is maintained in the presence of neoplastic glial cells has enabled assessment of invasive potential and has helped to elucidate the interaction between normal and neoplastic cells during invasion. In early studies, cell lines derived from ENU-induced brain tumours in the rat were confronted with embryonic chick heart fragments and this system has proved to be of value in assessing invasion of human brain tumours. Indeed, the degree of invasiveness in this system has been shown to correlate well with malignancy and clinical evolution of the neoplasms. For example, malignant gliomas invade into the normal tissue and destroy it while meningiomas surround but do not invade the "host" tissue. In other three-dimensional systems re-aggregated foetal brain has formed the target for *in vitro* invasion by both human and experimental animal brain tumour cell lines or short-term, early passage cultures maintained as either monolayer cultures or as multicellular tumour spheroids. Optic nerve, too, has provided a neural target with which to study invasion and has yielded information concerning the phagocytic activity of human and animal gliomas during invasion, as well as providing morphological evidence of interaction with the ECM.

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Cathepsins and cystatins in extracellular fluids – useful biological markers in cancer

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Proteases of all four classes have been shown to participate in processes of tumor growth, vascularisation, invasion and metastasis. Their levels in tumor tissue extracts can provide useful clinical information to predict disease free and overall survival in various types of cancer. Recently we found that cysteine proteinases cathepsins B and H and their endogenous protein inhibitors stefins A, B and cystatin C can also predict prognosis when measured extracellularly. In melanoma and colorectal cancer patients high serum levels of cathepsins as well as high levels of stefins A and B and cystatin C correlated with shorter survival. On the other hand, cathepsin B/cystatin C complex was found to be less abundant in sera of patients with malignant tumors than in those with benign diseases or in healthy controls.

Introduction

Alterations in expression, processing and localization of cysteine proteinases (CPs) in tumor tissues have been observed at various levels when compared to their normal and benign tissue counterparts.[1] CPs can be translocated to the plasma membrane or secreted from tumor cells where they presumably participate in the degradation of components of the extracellular matrix and basement membrane.[1,2] The mechanism of secretion is not fully understood[3], however, it is known that cathepsins can be secreted from normal and tumor cells as precursors or active enzymes. CPs may be involved also in the formation of new blood vessels, which enable feeding of the growing tumor.[4,5] Relation of mRNA, activity or protein levels of CPs in tumors with clinical characteristics of cancer patients has shown that these molecules are highly predictive for the length of survival and may be used for assessment of risk of relapse or death for cancer patients.[4] Detection of these proteins in extracellular fluids may extend their application to primary diagnosis[6-8], to the assessment of response to selected chemotherapy[9] and to the monitoring of malignant disease.

Quantization of cathepsins and their inhibitors in extracellular fluids

Cathepsins B, H and L have been determined in extracellular fluids of cancer patients by measuring enzymatic activity or immunochemically by ELISA. In first case various chromogenic and fluorogenic substrates and synthetic inhibitors have been used in experimental procedures, resulting in more or less specific signal for individual enzyme. In ELISAs specific monoclonal and polyclonal antibodies, raised to individual human antigens

have been used, providing reliable information of the protein level of each enzyme in extracellular fluids.[10,11] However, for most of cathepsins only the total protein level can be assessed by ELISA, excluding the information on active, pro or complexed enzyme forms. Only for detection of cathepsinB/cystatin C complex a specific ELISA has been designed.[11]

Extracellular levels of the inhibitors of CP have been defined by measuring total cysteine proteinase inhibitor (CPI) activity or by specific ELISAs.[13] Automated particle-enhanced immunoturbodimetric or immunonephelometric assays have been designed for detecting of cystatin C in blood.[12,13]

Extracellular cathepsins and their inhibitors as diagnostic or prognostic indicators

The activity and protein levels of cathepsins and inhibitors have been determined in fluids surrounding tumors, such as bronchoalveolar lavage fluid of lung cancer patients and ascites fluid of ovarian carcinoma patients and in blood and urine.[11]

High levels of cathepsin B have been reported in sera of patients with breast, ovarian, uterine, liver, pancreatic, melanoma, colorectal and lung cancer.[4,11] In patients with colorectal and uterine carcinoma cathepsin B protein or activity levels correlated with tumor stage.[8,14,15] Additionally, in this type of malignancy cathepsin B protein concentration was found to correlate with different modes of patient treatment.[16] Increased levels of cathepsin B were found in urine of patients with gastric cancer[17] but not in urine from breast 18 or bladder carcinoma.[19] Cathepsin B was found as a significant prognostic marker in sera of patients with melanoma[9] and colorectal cancer.[14] Patients with high levels of serum cathepsin B experienced high risk of death.

Cathepsin H was increased in sera of patients with melanoma, colorectal, lung and head and neck cancer.[11] In melanoma, its protein level was significantly increased within the group of patients who did not respond to the combined chemoimmunotherapy, compared with the group of responders, indicating the potential of this enzyme in predicting the effectiveness of the therapy.[9] In patients with head and neck cancer cathepsin H serum levels correlated with histological grade[20] and in melanoma and lung cancer its high levels correlated with shorter overall survival.[9,21]

Cathepsin L activity levels were found increased in sera of breast, pancreatic, liver and colorectal cancer.[11] Its protein level was found to be increased in sera of patients with ovarian carcinoma and suggested in combination with CA 125 and CA 72-4 as better marker for detection of ovarian cancer than the methods currently used in clinical practice.[7]

Stefin A and stefin B have been detected in ascitic fluid from ovarian carcinoma[22] and in bronchoalveolar fluid of lung cancer patients.[23] Increased serum levels of stefin A in patients with hepatocellular carcinoma and liver cirrhosis correlated with tumor size and with the number of neoplastic lesions.[6] Stefin A, but not stefin B levels were moderately increased also in sera of patients with colorectal and lung cancer.[24] Cystatin C was also increased in sera of cancer patients.[11] In melanoma, colorectal and lung cancer its levels correlated with the progression of the malignant disease. Since cystatin C has also been proposed as an accurate marker of glomerular filtration rate (GFR), its levels in cancer patients should be very carefully evaluated before clinical application of this new GFR marker. Stefin A, stefin B and cystatin C have been reported as significant prognostic markers in sera of patients with colorectal cancer.[24] High levels of all three inhibitors correlated with shorter overall survival although for stefin A the difference between high and low risk patients was not statistically significant. Stefin B was the strongest prognosticator of all three inhibitors and the combi-

nation with cathepsin B or CEA further stratified the risk of death.

In sera of patients with colorectal and lung cancer the level of cathepsin B/cystatin C complex was also determined.[11] The complex was significantly less abundant in sera of patients bearing malignant lung tumors than in those with benign lung diseases or in healthy controls. Similarly, in colorectal cancer sera, its level was lower in Dukes' stages C and D than in early stages A and B. The inverse correlation found in this study between malignant progression and stability of the complex, supports the hypothesis of hindered inhibitory capability during cancer progression.

Conclusions

Quantization of cysteine cathepsins and their inhibitors in extracellular fluids as compared with tumor tissue cytosols has many advantages. Besides prognostic information their levels can be used also for primary diagnosis, for the assessment of response to selected chemotherapy and for the monitoring of malignant disease. Additionally, the need for careful histological examination of tumor tissue, inherent problems with tissue heterogeneity and problems with the choice of extraction buffer do not apply to extracellular samples. On the other hand, the levels of cathepsins and their inhibitors in blood and other bodily fluids are much lower than in tissue extracts and their assessment requires more sensitive assays. Future activities should be focused on standardization and quality assurance of assays and on definition of subpopulations of cancer patients who would benefit most from the information provided by these new extracellular biological markers.

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Circadian rhythms of cysteine proteinases and cystatins, potential tumour markers, in normal sera

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Introduction

Circadian day/night variations have been evidenced in all major groups of organisms and at all levels of organisation of the organism. Circadian intra-individual variations are known for a number of analyses in serum including tumour-associated markers.[1-4] It was suggested that the serum levels of cysteine proteinases and their inhibitors may be of clinical importance for prognosis and diagnosis in cancer.[5] Since known circadian rhythms are important for choosing the best sampling time, interpretation of the results of a diagnostic test, patient monitoring, and timing of a therapy, our objective was to establish 24-h variations of cysteine proteinases, cathepsins B, H, L, and their low molecular weight inhibitors, stefin A, stefin B, and cystatin C, in sera from healthy subjects.

Materials and methods

This study, which included eight clinically symptom-free adults (median age, 31 years; range, 22-64 years; 5 females, 3 men), was performed as reported previously.[6,7] Before entering the study the volunteers had given informed written consent, and procedures were approved and performed in accordance with the guidelines of the regional medical ethics committee. Meals were served at 08:00, 12:30 and 18:00 h. The lights were switched off from 22:00 to 07:00 h.

Blood was taken by venipuncture in upright position according to National Committee for Clinical Laboratory Standards approved standard H3-A3. Seven samples were collected at 4-hour intervals beginning at 08:00 h. Blood was clotted at room temperature and centrifuged subsequently at 3000 rpm. Serum was separated, aliquoted and frozen at -20 °C until analysis.

Measurements of all proteins were done by specific ELISAs (Krka, d.d., Novo mesto, Slovenia) as described previously.[8,9]

Mean values and SE were computed at fixed hours for each subject during the 24-hour monitoring. All data were analysed by one-way ANOVA and by cosinor analysis involving the fit of a 24-hour cosine curve by the method of least squares[10,11] as reported previously.[6,7] The correlation between the parameters examined was assessed by the nonparametric Spearman rank correlation test. Two-sided P values < 0.05 were considered significant.

Results and discussion

The 24-h patterns of cathepsins B, H, L, cystatin C, stefins A and B were investigated in sera of clinically healthy subjects. To minimise the factors such as posture, activity, food ingestion, stress, sleep or wakefulness, which could contribute to the variations, the subjects were required to maintain the same regime during the study and 2 days before the beginning. All tested proteins in normal sera were in the nM concentration range and among them cystatin C was the most abundant since only cystatin C is localised extracellularly (Table 1). Common feature of 24-hour patterns was that cystatins and cathepsins reached their minimal values in the resting period, except for cathepsin B and stefin B, which were close to the daily mean throughout the day. Comparing all patterns of cathepsins and cystatins a significant relationship between the variations was observed only for cathepsins H and L, indicating possible similar regulation of expression.

To eliminate between individual variability, each individual's data were transformed to deviation from that individual's 24-hour mean. All data were subjected to ANOVA, which validated any apparent differences by comparing different time points. Since ANOVA can fail to obtain the actual high point of the rhythm, data were analysed also individually and as a group for circadian rhythm by single and population mean cosinor analysis, which involves the fit of a 24-hour cosine curve by the method of least squares. The method provides both the probability of rejection of the zero amplitude hypothesis for a chosen period (24 h in our case) and rhythm characteristics: the mesor (24-hour adjusted means), the amplitude (half the difference between the maximum and the minimum fitted cosine function), and the acrophase (time of maximum in fitted cosine function, with midnight as the phase reference). ANOVA revealed no significant time effect except for transformed data of cathepsins H and L and both stefins (Table 1). Using single cosinor analysis no significant rhythm was revealed, except for cystatin C, stefin A and cathepsin H, where only one subject demonstrated a circadian variation ($P \leq 0.04$). On the group level using population mean cosinor analysis, the patterns of all investigated serum prote-

Table 1. Circadian characteristics for serum cathepsin B (CB), cathepsin H (CH), cathepsin L (CL), cystatin C (CC), stefin A (SA) and stefin B (SB), measured every 4 hours for 24 hours in healthy subjects. 24-h means with range between subjects are presented. Statistical evaluation for circadian time effect and rhythm was determined by ANOVA and cosinor analysis.

				ANOVA			**Least-squares fit of 24-h cosine**		
Serum protein	**Units**	**24-h mean ± 2 SE**	**Range**	**F**	**P**	**P**	**Mesor ± SE**	**Amplitude ± SE**	**Acrophase ± SE (h)**
CB	ng/ml	4.5 ± 0.5	3.5 – 7.5	0.02	1.0	0.7	4.5 ± 0.5	0.1	08:40
	% of mean	19 ± 2	11 – 26	0.4	0.9	0.8	100 ± 0.2	1	06:55
CH	ng/ml	15.0 ± 2.2	7.8 – 24.4	0.4	0.9	0.1	14.8 ± 2.2	1.6	11:51
	% of mean	112 ± 51	18 – 440	2.8	0.02	0.2	99 ± 1	15	11:13
CL	ng/ml	18.1 ± 2.7	7.5 – 32.3	0.5	0.8	0.02	18.0 ± 2.7	2.1 ± 0.5	11:38 ± 00:41
	% of mean	66 ± 17	7 – 113	6.6	<0.001	0.03	99 ± 0.3	15 ± 4	12:01 ± 00:47
CC	ng/ml	681.9 ± 39.4	438.2 – 1156.0	0.4	0.9	0.2	675.5 ± 76.2	40.2	07:19
	% of mean	60 ± 9	27 – 90	1.9	0.1	0.2	99 ± 0.3	5	07:23
SA	ng/ml	6.1 ± 0.4	4.2 – 8.2	1.0	0.4	0.2	6.1 ± 0.4	0.3	14:26
	% of mean	85 ± 7	32 – 77	3.1	0.01	0.2	100 ± 1	6	14:34
SB	ng/ml	3.0 ± 1.2	0.5 – 8.8	0.05	1.0	0.1	3.0 ± 1.2	0.1	06:52
	% of mean	49 ± 7	19 – 86	4.3	0.002	0.2	100 ± 0.3	5	03:46

ins showed no significant circadian rhythm with the exception of cathepsin L where the rhythm exhibited a small amplitude, ranging from 5-24 % of the 24-hour mean, and an acrophase localised at around 12 h (Table 1).

Conclusion

We conclude that the time of sampling in the course of day has a minor influence on measurements of cathepsin L, and none on cathepsins B and H, stefins A and B, and cystatin C in normal sera which underlines their usefulness as potential clinical markers. The possible changes in their circadian structure with different types of cancer will be of considerable interest.

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Immunohistochemical analysis of cathepsin B and cathepsin S in tumors, parenchyma and regional lymph nodes of the lung

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Introduction

The detection of antigens on tissue sections by using specific antibodies (immunohistochemistry (IHC)) is an important approach in identifying cell types, which express the specific antigens. Although the immunohistochemical analysis (IHA) is a relatively well established and simple method, the major problem remains to detect low concentrations of specific antigens. In this respect, the efforts are mainly focused on improved detection systems after binding of the specific primary antibody.

Cathepsin (cath) S is present at rather low levels in lung tissue extracts[1], therefore low expression of cath S at the cellular level is expected. In fact, cath S was hardly detected by using standard procedures described in the literature. To solve this problem we established a highly sensitive staining protocol based on the CSA detection system from DAKO (Hamburg, Germany) in combination with antigen retrieval approach.

Material and methods

In order to identify cell populations producing cath S, we performed IHC on tissue sections deriving from formalin-fixed, paraffin-embedded tumors (n = 36), non-tumors parenchyma (n = 6) and regional lymph nodes of the lung (n = 10). In addition, the expression of cath S in lung cell populations was compared with those of cath B.[2] Furthermore, the expression of both cysteine-cathepsins was correlated with clinical-pathological parameters of lung cancer patients.

Results

Cath S is expressed in alveolar type II cells, macrophages, bronchial epithelial cells and lymphocytes. The enzyme showed a lysosomal distribution over the cytoplasm. In addition, we observed differences in the staining intensity of cath S in macrophages, alveolar type II cells and lymphocytes, as well. In some bronchial epithelial cells a more restricted localization in the basal proliferating zone of the epithelium was observed. Immune reactive cath S was not found in alveolar type I cells. However, cath S could also be detected in lung tumors, independently of their origin. We found a positive reaction in adenocarcinomas, squamous cell carcinomas and small cell carcinomas. Remarkably, a weak positive reaction at sides of interaction between tumor cells of the small cell carcinoma and the extracellular matrix of the alveoli could be observed. It should be noted that also in alveolar duct cells a focal positive reaction was visible.

Cath B could easily be detected using a standard protocol in combination with the ABC-system (Vector Laboratories, Serva Heidelberg), but without the need of an additional enhancing step. This clearly indicates that

cath B is expressed at much higher levels in various cell populations compared with cath S. Cath B could also be detected in alveolar type II cells, macrophages, bronchial epithelial cells and various tumors. In contrast to cath S, we were not able to localize cath B in lymphocytes. The expression of cath S did not correlate with clinical-pathological parameters of lung cancer patients, while for cath B ΣpN1/PnP/pN3 primary tumors were more frequently labelled than PnP tumors.

Conclusion

We established a highly sensitive IHC protocol for the detection of cath S in tissue sections deriving from tumors, parenchyma and regional lymph nodes of the lung. Our results show that of the two cysteine cathepsins (B and S), only cath S seems to be produced by lymphocytes, This indicates that cath S may be involved in regulatory mechanisms of the immune response.

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Characterization of monoclonal antibodies against MHC class II-associated p41 invariant chain fragment

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Mouse monoclonal antibodies directed against human MHC class II-associated p41 invariant chain fragment have been generated. Mice were immunized with human recombinant Ii-isoform p26. For hybridoma production mouse splenocytes and myeloma cells were fused. Hybridoma cells were screened using ELISA and immunoblotting. Three cell lines (42B10, 42G11 and 43C8) were used for production of specific antibodies, which reacted with p41 fragment and did not bind to cathepsins L or S or their proenyzmes. As primary antibody for immunofluorescence staining of lymph node tissue sections clone 2C12 MAb was selected. Specific localization of p41 fragment in certain cells in lymph nodes was observed.

Introduction

MHC class II molecules display antigenic peptides on cell surface of APC (dendritic cells, B cells, macrophages, thymic epithelial cells) for recognition by $CD4^+$ T lymphocytes.[1,2,3] The MHC class II-associated Ii is a transmembrane protein that complexes with newly synthesized MHC class II heterodimers and directs their trafficking through the endosomal compartments of APC. The luminal domain of Ii organizes MHC class II dimers into nonomeric complexes and prevents premature association of MHC class II molecules with endogenous polypeptides. Within endosomal/lysosomal compartments, Ii undergoes stepwise proteolytic degradation to yield progressively smaller fragments that remain associated with the peptide-binding groove of MHC class II dimmers.[4] Dissociation of CLIP (a set of 3 kDa peptides) from the peptide-binding groove allows loading and subsequent surface expression of MHC class II molecules with antigenic peptides generated from endocytosed or phagocytosed protein. The key enzymes that degrade Ii are the cysteine proteases (cathepsins S[5], L[6] and/or V[7]). In human Ii exists in two alternatively spliced forms, p31 in p41[3], the latter containing an additional 64-amino acid sequence at the C-terminal end (hereafter called the p41 fragment). The discovery of p41 fragment-cathepsin L complex isolated from human liver[8] and its known crystal structure[9], led to the suggestion that the p41 fragment (Ii respectively) may enhance antigen presentation by providing a mechanism to inhibit otherwise destructive cathepsin L but not cathepsin S activity.[10] The aim of the present study was to generate specific mouse monoclonal antibodies directed against MHC class II-associated p41 invariant chain. Furthermore these antibodies were used in immunohistochemical studies of p41 fragment in lymph-node tissue.

Materials and methods

Preparing and purification of antibodies

Human recombinant Ii-isoform p26, comprising only the luminal domain[11] (together with p41 fragment) was purified by Ni-chelate chromatography (a gift from Dr. Klaus Dornmair, Max Planck Institute, Martinsried) and was subsequently used as an antigen for immunization of mice. BALB/c mice were injected subcutaneously with p26 (50 μg/mouse) emulsified in complete Freund's adjuvant, followed by intraperitoneal injections of the same amount of antigen in incomplete Freund's adjuvant. Test bleeds were taken and titer of specific antibodies determined using antigen immobilized ELISA in which recombinant p26 and p41 fragment-cathepsin L complex, respectively, were used as antigens. The mouse with the highest titer was boosted intraperitoneally with p26 (50 μg/mouse). For hybridoma production splenocytes and myeloma cells (NS1/1-Ag4-1) were fused by a modification of the method of Kohler and Milstein.[12] The screening of the positive wells was performed by antigen immobilised ELISA as described above for test bleeds. Hybridomas producing antibodies against p41 fragment were cloned twice by means of the limiting dilution method[13] and expanded into large volumes. The cell culture supernatants were concentrated by ultrafiltration and MAbs purified by affinity chromatography on Protein A-Sepharose (Pharmacia, Sweden). Antibody containing fractions were pooled and dialysed against PBS, pH 7.2. Small aliquots of purified antibodies were stored at – 20 °C.

Immunoblotting

Samples were first separated by SDS-PAGE on 8 – 25 % polyacrylamide gels using Phast-System (Pharmacia, Sweden). After the electrophoresis the proteins were transferred onto PVDF membrane (Millipore, USA) by passive diffusion accelerated with higher temperature. Non-specific binding sites were blocked with 0.4 % Tween 20 in PBS, pH 7.2. After this and all subsequent steps the membrane was washed with PBS, pH 7.2 containing 0.5 % Tween. Primary anti-p41 fragment antibodies were incubated with the membrane, followed by secondary goat anti-mouse IgG conjugated to HRP (Dianova, Germany). Detection was performed using 0.05 % DAB (Sigma, USA) and 0.01 % H_2O_2 in 50 mM Tris-HCl buffer, pH 7.5.

Immunofluorescence tissue staining

Sections from formalin fixed, paraffin embedded lymph nodes were used for IHA. Tissue sections on micro cover glasses were deparaffinised in xylene and rehydrated through ethanol series. They were placed in 10 mM sodium citrate buffer, pH 6.0 and put into a microwave oven (5 min, 400 W) for antigen retrieval. Non-specific binding sites were blocked with 3 % BSA in PBS, pH 7.4. After this and all subsequent steps tissue sections were rinsed in PBS, pH 7.4. Primary anti-p41 fragment antibody was added (clone 2C12, 25 μg/ml, for 2 h at 37 °C), followed by Alexa Fluor™ 488-labeled goat anti-mouse IgG secondary antibody. Molecular Probes, USA). Tissue sections were mounted on slides with ProLong™ AntiFade Kit (Molecular Probes, USA). Fluorescence microscopy and optical slicing were performed by confocal laser scanning microscope LSM 510 (Carl Zeiss Inc., USA).

Results

Purified monoclonal antibodies were tested for specificity using immunoblotting. As shown in Figure 1, 2C12 MAb (derived from clone 42B10) reacted with p41 fragment in complex with cathepsin L (32 kDa, lane 5),

with p41 fragment detached from the complex (14 kDa, lane 1 and 2), as well as with recombinant p26 (lane 3). There was no cross-reactivity observed with recombinant procathepsin L (lane 4), cathepsin L (31 kDa, lane 1) nor its heavy chain (25 kDa, lane 1) and light chain (below 14 kDa, lane 1), respectively.

Also, antibody specificity and cross-reactvity towards different cathepsins was tested by ELISA. Recombinant (pro)cathepsins L and S were added to the wells instead of p26 or p41 fragment-cathepsin L complex. With all three selected cell lines negligible reactivity was observed. For immunohistochemical localization of p41 fragment in lymph node tissue sections 2C12 MAb was selected. Results are shown in Figure 2.

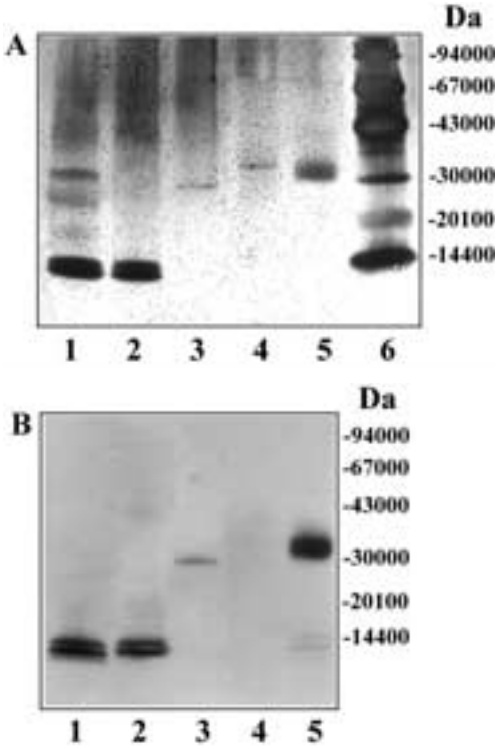

Figure 1. Demonstration of the specificity of anti-p41 fragment Mab. (A) SDS-PAGE silver staining. (B) Immunoblot of the equivalent gel stained with 2C12 MAb. Samples: (lane 1) p41 fragment-cathepsin L complex reduced (with 5% 2-mercaptoethanol) and exposed to 100 °C in the presence of SDS for 5 minutes; (lane 2) p41 fragment detached from the native complex by HPLC; (lane 3) p26 reduced (with 5% 2-mercaptoethanol) and exposed to 100 °C in the presence of SDS for 5 minutes; (lane 4) nonreduced recombinant procathepsin L; (lane 5) nonreduced p41 fragment-cathepsin L complex; (lane 6) LMW standards.

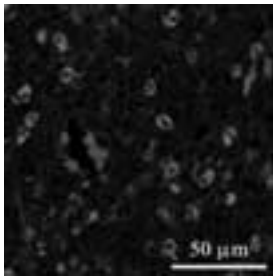

Figure 2. Immunohistochemical staining of lymph-node tissue sections for p41 fragment.

Conclusions

Specific monoclonal antibodies recognizing MHC class II-associated p41 fragment were successfully produced. They do not cross react with cathepsins L nor S or their proenzymes. We have shown specific localization of Ii fragments in certain cells in lymph nodes. These antibodies provide new tools for investigating subcellular colocalization of Ii together with cathepsins S and L.

Acknowledgements

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In vitro and *in vivo* angiogenic assays

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The formation of new vessels in a tumour requires a number of steps including chemotaxis, migration, proliferation and tubular formation of the endothelial cells. Thus, in vitro assays, which dissect out each of these steps or a combination of these, can be designed. For example, tubular formation assays can be conducted using collagen gels as matrix and angiogenic factors such as VEGF and bFGF as stimuli. In addition to studying these functions of the endothelial cells under various conditions, these assays can also be used to test the importance of different molecules including molecules with a potential inhibitory effect on tumour angiogenesis. The next question that arises is which endothelial cells to use. HUVEC (human umbilical vein endothelial cells) can be obtained from commercial sources and these cells can grow for approximately 15 to 20 passages. Another possibility is to establish primary cultures of endothelial cells. We have used this technique to establish primary lung endothelial cell cultures from wild-type mice and from knock-out mice. The resulting cell lines can then be compared using some of the above-mentioned in vitro assays. A large number of in vivo angiogenesis assays have been described. The more common ones are implantation of bFGF or VEGF pellets either just subcutaneously, in a dorsal air sack or embedded in Matrigel. Other models include wound healing, retina damage etc. However, caution should be taken regarding extrapolating results from assays including non-malignant conditions to tumour angiogenesis. The mediators of tumour angiogenesis may very well be different from those mediating angiogenesis in non-tumour conditions.

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In vitro and *in vivo* angiogenic assays

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The formation of new vessels in a tumour requires a number of steps including chemotaxis, migration, proliferation and tubular formation of the endothelial cells. Thus, in vitro assays, which dissect out each of these steps or a combination of these, can be designed. For example, tubular formation assays can be conducted using collagen gels as matrix and angiogenic factors such as VEGF and bFGF as stimuli. In addition to studying these functions of the endothelial cells under various conditions, these assays can also be used to test the importance of different molecules including molecules with a potential inhibitory effect on tumour angiogenesis. The next question that arises is which endothelial cells to use. HUVEC (human umbilical vein endothelial cells) can be obtained from commercial sources and these cells can grow for approximately 15 to 20 passages. Another possibility is to establish primary cultures of endothelial cells. We have used this technique to establish primary lung endothelial cell cultures from wild-type mice and from knock-out mice. The resulting cell lines can then be compared using some of the above-mentioned in vitro assays. A large number of in vivo angiogenesis assays have been described. The more common ones are implantation of bFGF or VEGF pellets either just subcutaneously, in a dorsal air sack or embedded in Matrigel. Other models include wound healing, retina damage etc. However, caution should be taken regarding extrapolating results from assays including non-malignant conditions to tumour angiogenesis. The mediators of tumour angiogenesis may very well be different from those mediating angiogenesis in non-tumour conditions.

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Nepopolna spontana raztrganina uretra. Prikaz primera

Borković Z, Srdoč D, Bedalov G

Izhodišča. Avtorji so v prispevku predstavili primer spontane delne raztrganine uretra med napadom ledvičnih kolik, ki ga je povzročil ledvični kamen v proksimalnem delu uretra v višini processus transversusa L3.
Prikaz primera. Urografsko slikanje je pokazalo razširitev ureteralnega kanala pa tudi ledvičnega meha. V višini pielo-ureternega prehoda je slika pokazala iztekanje kontrastnega sredstva. Kontrastno sredstvo je bilo razlito ob musculusu psoas. Prečne slike, narejene z računalniško tomografijo, so pokazale razlito kontrastno sredstvo vzdolž medialnega in dorzalnega dela perirenalnega prostora in distalno ob musculus psoas. Ureter je bil na sliki prikazan od raztrganine na pielo-ureternem prehodu do mesta ledvičnega kamna. Na uretru ni bilo znamenj o poškodbah parenhima ali o krvavitvah v perirenalnem prostoru. Med operacijo smo našli mesto raztrganine, ki je bilo obdano z veliko količino perirenalne in periureterne tekočine. Po odstranitvi kamna smo raztrganino kirurško zašili. Pooperativna urografija in slika uretra z računalniško tomografijo sta bili normalni.
Zaključki. Pri delni spontani raztrganini uretra še vedno vidimo v uretru kontrastno sredstvo distalno od mesta raztrganine in iztekajoče vzdož musculusa psoas.

Radiol Oncol 2002; 36(2): 91-4.

Gastropareza pri mladi bolnici s sladkorno boleznijo. Prikaz primera

Kovačič P, Jamar B

Izhodišča. Gastropareza je upočasnjeno praznjenje želodca in se pojavlja pri različnih bolezenskih stanjih, na primer po vagotomiji ali pri sistemskih boleznih kot so sladkorna bolezen, sklerodermija in amiloidoza. Namen tega članka je predstaviti rentgensko preiskavo, ki je enostavna, zanesljiva in neinvazivna, kot alternativno metodo drugim metodam za oceno praznjenja želodca.
Prikaz primera. Enaindvajsetletna ženska je bila sprejeta zaradi suma na avtonomno nevropatijo. Zadnjih deset let je imela od insulina odvisno sladkorno bolezen tipa I. Ob sprejemu je opisovala nauzejo, bruhanje, oslabelost in občasne vrtoglavice. Pri rentgenski preiskavi z barijem je bil viden dilatiran, aperistaltičen požiralnik, v aperistaltičnem želodcu so bili ostanki hrane, in nobene strukturne spremembe, ki bi povzročala oviro praznjenja želodca, ni bilo videti.
Zaključki. Scintigrafija trenutno velja za standardno preiskavno metodo za oceno praznjenja želodca. Želodčno motoriko pregledujejo še z ultrazvokom, elekrogastrografijo in antroduodenalno manometrijo, praznjenje želodca pa ocenjujejo tudi z magnetno rezonanco in radiopačnimi markerji.Vloga rentgenske preiskave z barijem še ni dokončno razjasnjena.

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Radiol Oncol 2002; 36(2): 95-102.

Magnetna resonančna spektroskopija. Pregled metode in njena uporaba v klinični neuroradiologiji

Koren A

Izhodišča. Magnetno resonančna spektroskopija (MRS) je sorazmerno nova diagnostična metoda. Možganovina je zelo primerno tkivo za te vrste analizo. V praksi pa je z MRS mogoče analizirati le majhno število sestavin, ki se so v možganskem tkivu. Uporabnost metode v neuroradiologiji in pri kliničnem delu narašča, saj pomaga tako pri diferencialni diagnostiki patoloških procesov kot pri opredelitvi progresije bolezni oz. uspeha terapije. Pri analizi rezultatov preiskave je potrebno upoštevati številne dejavnike, ki lahko vplivajo na objektivnost izvida. Magnetno resonančni tomograf na kliničnem inštitutu za radiologijo v KC v Ljubljani omogoča sodobne MRS protokole, ki jih uporabljamo v diagnostiki nevroloških in drugih bolezni.
Zaključki. MRS omogoča spektralno analizo snovi v izbranem volumnu tkiva in s tem vpogled v njegovo metabolno stanje.

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Napredki v s kontrastom ojačani MR-angiografiji: Indikacije in omejitve

Aschauer MA, Stollberger R, Ebner F

Izhodišča. Z gadolinium(Gd)-ojačana tri-dimenzionalna (3D) magnetno resonančna angiografija (MRA) je novejša tehnika, s katero hitro dobimo podatke visoke ločljivosti tako za prikaz arterij kot ven v celem telesu.
Zaključki. S kontrastom ojačana 3D MRA predstavlja mejnik za neinvaziven slikovni prikaz žilja. Metem, ko je bila klinična uporabnost 3D MRA že dokazana v številnih področjih žilne diagnostike, bo stalen razvoj strojne in programske opreme, kakor tudi novih kontrastnih sredstev pripeljal do nadaljnega širjenja indikacij. Rutinsko lahko opravljamo 3D prikaz ledvic, ureterjev in mehurja, s čimer lahko prikažemo in ocenimo zaporo, zapoznelo funkcijo, polnitvene defekte in mase v predelu ledvic. Bolniki z vzpodbujevalniki srca, niso primerni za preiskavo z MRA, nekatere vrste endoprotez in imobilizacijskih sredstev pa povzročajo pomembne artefakte, ki skrijejo pomembne. Ločljivost CE-3D-MRA je nižja v primerjavi z konvencionalno angiografijo, omejena pa je tudi ločljivost majhnih perifernih arterij.

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Poškodbe normalnega tkiva zaradi radioterapije in kemotearpije: vloga citokinov in adhezijskih molekul

Plevová P

Ozadje. Ionizirajoče sevanje in citostatiki, ki jih uporabljamo za zdravljenje raka, poškodujejo normalno tkivo in sprožijo celovit odgovor tako na celularni kot molekularni ravni, vanj pa so vpleteni tudi citokini in adhezijske molekule.
Metode. Zbrali in pregledali smo že objavljene podatke.
Rezultai in zaključki. Različni citokini in adhezjske molekule, kot so nekrotizirajoči faktor alfa interleukini 1, 2, 4 in 6, intereferon gama, granulocite in makrofage stimulirajoči faktor, transformirajoči rastni faktor beta, faktor aktiviranja krvnih ploščic, intercelična adhezijka molekula 1, vaskularno-celična adhezijska molekula- 1, in selektini E in P so vpleteni v odgovor normalnega tkiva na zdravljenje z ionozirajočim sevanjem in kemoterapijo in so odgovorni za poškodbe tkiva zaradi zdravljenja ter nezaželenih učinkov teh načinov zdravljenja, na primer vročine, anoreksije, utrujenosit, zaviranja hematopoeze ter akutnega in poznega lokalnega odziva na zdravljenje.

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Učinkovitost trastuzumaba in paclitaxela pri zdravljenju bolnic z metastatskim rakom dojke in izraženim HER-2/neu onkogenom

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Izhodišče. Trastuzumab je poznan kot učinkovito zdravilo pri bolnicah z rakom dojke in izraženem HER2/neu onkogenom. V prospektivni študiji smo proučevali učinkovitost, varnost in toksičnost trastuzumaba in paclitaxela pri metastatskem raku dojke, ki je bil v progresu po predhodnem zdravljenju.

Bolniki in metode. Vključili smo 17 bolnic z histološko potrjenim rakom dojke, s stanjem zmogljivosti po Karnofskem vsaj 60 %, srednjo starostjo 50 let (36-66), ki so bile prej zdravljene z vsaj dvema shemama kemoterapije. Izražanje HER-2/neu smo preverjali z Herceptest ® (DAKO) pri vseh 17 bolnicah. Petnajst vzorcev je bilo 3+ positivnih, dva vzorca pa 2+ positivna. Vse bolnice razen ene so bile prej zdravljene s taksani. Interval brez taksanov (TFI) smo definirali kot čas med zadnjo aplikacijo taksanov in pričetkom študije za vsako bolnico posebej. TFI je bil daljši od enega leta pri 7 bolnicah, TFI krajši od enega leta pa pri 9. Začetna doza Trastuzumaba je bila 4 mg/kg i.v., nato pa 2 mg/kg i.v. vsak teden. Paclitaxel smo aplicirali v dozi 80 mg/m^2 i.v. vsak teden do napredovanja bolezni ali pa nesprejemljive toksičnosti. Ocenjevali smo stopnjo odgovora (RR), čas do napredovanja bolezni (TTP), preživetje (OS) in toksičnost.

Rezultati. V populaciji z namenom zdravljenja smo ugotovili objektiven odgovor pri 10 bolnicah (59 %), vključno z dvema popolnima odgovoroma (CR). V podskupini z TFI > 1 leto smo ugotovili odgovor v štirih primerih, vključno z enim CR (RR 57 %). V podskupini z TFI < 1 leto pa smo ugotovili odgovor v 6 primerih vključno z enim CR (RR 67 %). TFI ni bil statistično pomemben za odgovor ($p < 0,4349$). Srednji TTP znaša 6 mesecev , 4 bolnice pa so še vedno brez progresa. Bolnice z TFI > 1 leto imajo daljši TTP ($p = 0,0201$). Pri 10 živih bolnicah nismo dosegli srednjega OS. Aplicirali smo 599 krogov terapije vključno z 473 krogi trastuzumaba in paclitaxela brez prilagajanja odmerka. Ena bolnica je pri prvi aplikaciji trastuzumaba razvila preobčutljivostno reakcijo in smo jo izključili iz študije. Najpogostejši toksični učinek je bila z infuzijo trastuzumaba povezana piretična reakcija, ki smo jo opazili pri šestih bolnicah. Edini stranski učinek, ki je pripeljal do prekinitve zdravljenja je bila kardiotoksičnost. Zmanjšanje iztisne frakcije druge stopnje se je pojavilo pri eni bolnici, ravno tako pa tudi tretje stopnje. Šest bolnic je izkusilo nevropatijo tretje stopnje. Pri eni bolnici se je pojavila nevtropenija četrte stopnje in anemija tretje stopnje. Ugotovili smo štiri primere infekcije tretje stopnje brez nevtropenije. Poslabšanje testov jetrne funkcije tretje stopnje smo ugotovili pri šestih bolnicah, niso pa zahtevali prilagoditve odmerka. Ugotovili smo še en primer hiperglikemije tretje stopnje in en primer povečanja telesne teže tretje stopnje.

Zaključki. Trastuzumab in paclitaxel sta pokazala učinkovitost in dobro toleranco pri bolnicah z metastatski mrakom dojke z prekomernim izražanje HER-2/neu. Odgovor tumorja pri desetih bolnicah, ki so so bile prej zdravljene s taksani in so odgovorile na zdravljenje ni bil odvisen od TFI, so pa bolnice z daljšim TFI imele daljši TTP.

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Naravni inhibitorji proteaz v tumorju

Magdolen U, Krol J, Sato S, Mueller MM, Sperl S, Krüger A, Schmitt M, Magdolen V

Preoblikovanje zunajceličnega matriksa je osnova mnogih normalnih bioloških procesov, na primer razvoja, morfogeneze in celjenja rane. Preoblikovanje zunajceličnega matriksa opazimo tudi pri zelo resnih patoloških okvarah, na primer pri aterosklerozi, fibrozi, invazivnosti tumorja in razvoju metastaz. V takšno preoblikovanje so najpogosteje vpletene serinske proteaze (še zlasti plazminogenski aktivator-urokinaza / plazminski sistem), metaloproteaze matriksa (družina približno 20 od Zn odvisnih endopeptidaz, vključno s kolagenazami, želatinizami, stromelizini in metaloproteazami membranskega tipa) ter cisteinske proteaze. Dejavnost teh proteaz in vivo v zunajceličnem prostoru uravnavata aktiviranje zimogena in nadzorovana inhibicija. V preglednem članku predstavljamo zgradbo in biokemične lastnosti pomembnih inhibitorjev proteaz, na primer inhibitor plazminogenskega aktivatorja tipa 1 in 2 (PAI-1 in PAI-2,) tkivnih inhibitorjev metaloproteinaz (TIMP-1, -2, -3 in -4) in inhibitorjev cisteinskih proteaz cistatin C, ki so povezani z razvojem tumorja. Zanimivo je, da nekateri od teh inhibitorjev tumorskih proteaz opravljajo hkrati več nalog, ki pravzaprav bolj pospešujejo kot zavirajo napredovanje tumorja, če prisotnost inhibitorjev v tumorskem tkivu ni uravnoteženea.

Radiol Oncol 2002; 36(2): 145-51.

Inhibitorja cisteinskih proteinaz stefin A in stefin B pri operabilnem karcinomu glave in vratu

Strojan P, Budihna M, Šmid L, Svetic B, Vrhovec I, Kos J, Škrk J

Namen. Ovrednotiti vlogo inhibitorjev cisteinskih proteinaz stefinov A in B v procesu odločanja o zdravljenju in njihov napovedni pomen pri operabilnem ploščatoceličnem karcinomu glave in vratu.

Bolniki in metode. Koncentracije stefinov A in B so bile izmerjene imunobiokemično z uporabo ELISA testov v citosolih, pripravljenih iz tkiva tumorja in okolne zdrave sluznice 91 bolnikov z operabilnim ploščatoceličnim karcinomom glave in vratu. Ob zaključku opazovanega obdobja je znašal srednji čas spremljanja preživelih bolnikov 5,8 let (razpon 5-9,3 leta).

Rezultati. Koncentracija stefina A je bila statistično pomembno višja v vzorcih tumorja kot v vzorcih zdrave sluznice ($P = 0.05$). V skupini bolnikov s klinično tipnimi bezgavkami pred zdravljenjem ($n = 57$) je bila ugotovljena signifikantna razlika v koncentracijah stefina A ($P = 0.03$) in stefina B ($P = 0.02$) med tistimi s histopatološko potrjeno prizadetostjo bezgavk in tistimi z neprizadetostjo vratnih bezgavk. V univariatni analizi preživetja so se kot prognostično ugodnejše izkazale visoke koncentracije stefinov. Stefin A je potrdil svoj neodvisen napovedni pomen tudi v multivariatni analizi.

Zaključki. Kot dejavnika, zmožna razlikovati med patološkima stadijema bolezni pN_0 in pN_+ pri bolnikih s klinično ugotovljeno prizadetostjo bezgavk, bi stefina A in B lahko vplivala na odločitev o obsegu operacije na vratu. Oba stefina sta se izkazala kot zanesljiva kazalca za napoved preživetja bolnikov z operabilnim ploščatoceličnim karcinomom glave in vratu.

Notices

Notices submitted for publication should contain a mailing address, phone and/or fax number and/or e-mail of a ***Contact*** *person or department.*

CT scanning

June 13-16, 2002

The meeting "7th Annual Computed Body Tomography for the Technologist" will take place in Las Vegas, Nevada, USA.

Contact Conference Co-ordinator, Office of Continuing Medical Education, Johns Hopkins University School of Medicine, Turner 20/720 Rutland Avenue, Baltimore, Maryland 21205-2195, USA; or call +1 410 955 2959; or fax +1 410 955 0807; or e-mail cmenet@jhmi.edu; or see http://www.med.jhu.edu/cme

Bronchology and bronchoesophagology

June 16-19, 2002

The "12th World Congress for Bronchology" and the "12th World Congress for Bronchoesophagology" will be offered in Boston, USA.

Contact Congress Secretariat. Tufts University School of Medicine. Office of Continuing Education, 136 Harrison Avenue, Boston, MA 02111, USA, or call +1 617 636 6509; or fax +1 617 636 0472; or see http://www.aabronchology.org

Brachytherapy

June 16-20, 2002

The ESTRO teaching course "Modern Brachytherapy Techniques" will take place in Lisbon, Portugal.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Clinical oncology

June 21-22, 2002

The "3rd International Anglo-Croatian Symposium on Clinical Oncology" in collaboration with "51 Radiotherapy Club" (UK) meeting will be offered in Dubrovnik Cavtat, Croatia.

Contact Dr. Fedor Šantek, Executive Secretary; Medical school, Clinic of Oncology and Radiotherapy, University Hospital Centre Rebro, Kišpatičeva 12, Zagreb, Croatia; or call +385 1 4552 333.

Radiotherapy

June 23-27, 2002

The ESTRO teaching course "IMRT and Other Conformal Techniques in Practice" will take place in Amsterdam, The Netherlands.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Radiotherapy

June 23-27, 2002

The ESTRO teaching course "Imaging for Target Volume Determination in Radiotherapy" will take place in Coimbra, Portugal.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Oncology

June 30 – July 5, 2002

The "18th UICC International Cancer Congress" will be offered in Oslo, Norway.

Contact Norwegian Cancer Society, P.O. Box 5327 Majorstua, N-0304 Oslo, Norway, or call +47 22 59 30 00; or fax +47 22 60 69 80; or e-mail cancer@oslo2002.org

Radiology

July 1-5, 2002

The "22nd International Congress of Radiology (ICR 2002)" will take place in Cancun, Mexico.

Contact B.P. Servimed, S.A. de C.V., at Insergentes Sur No. 1188 50 piso, Col. Del Valle, 03210 Mexico DF, Belgium; or call +525 575 9931; or fax +525 559 9407; or e-mail fmricr@servimed.com.mx

Oncology

July 3-5, 2002

The ESO course "Cancer Economics and Evidence-Based Medicine" will take place in Sapporo, Japan.

Contact ESO Office, Viale Beatrice d'Este 37, 20122 Milan, ltaly; or call +39 02 43359611; or fax +39 02 43359640; or e-mail esomi@tin.it; or see http://www.cancerworld.org

Biomedical spectroscopy

July 7-10, 2002

The "First International Conference on Biomedical Spectroscopy: From Molecules to Men" will take place in Cardiff, Wales, United Kingdom.

Contact Dr Parvez I. Haris, Department of Biological Sciences, De Montfort University, The Gateway, Leicester, LE1 9BH, United Kingdom; or call +44 116 2506306; or fax +44 116 2577287; or e-mail: pharis@dmu.ac.uk; or see http://www.dmu.ac.uk/in/biospectra/

CT scanning

July 25-28, 2002

The "10th Annual Advanced Topics in CT Scanning: The 2002 Edition" will take place at Lake Tahoe, NV, USA.

Contact Conference Co-ordinator, Office of Continuing Medical Education, Johns Hopkins University School of Medicine, Turner 20/720 Rutland Avenue, Baltimore, Maryland 21205-2195, USA; or call +1 410 955 2959; or fax +1 410 955 0807; or e-mail cmenet@jhmi.edu; or see http://www.med.jhu.edu/cme

Clinical Oncology

August 4-9, 2002

The "Masterclass in Clinical Oncology" will take place in Montecatini Terme, Italy.

Contact Dr. Wolfgang Gatzermeier, ESO Office, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 0258317850; or fax +39 02 433 59640; or e-mail esoweb@tin.it

Radiation physics

August 25-29, 2002

The ESTRO teaching course "Physics for Clinical Radiotherapy" will take place in Leuven, Belgium.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Radiobiology

August 25-29, 2002

The ESTRO teaching course "Basic Clinical Radiobiology" will take place in St. Petersburg, Russia.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Oncohaematology

August 29-30, 2002

The ESO course will take place in Buenos Aires, Argentina.

Contact G. Farante, ESO Headquarters, ESO Latin America Office, Viale Beatrice d'Este 37, 20122 Milan, ltaly; or call +39 02 58317318; or fax +39 02 58321266; or e-mail esolatin@tin.it; or see http://www.cancerworld.org; or Argentina Office, A. Rancati, Florida 833 (1o), 1005 Buenos Aires; Phone +54 11 45118078; Fax +54 11 45118079.

Oncohaematology

August 31 – September 1, 2002

The ESO course will take place in Bahia, Brazil.

Contact G. Farante, ESO Headquarters, ESO Latin America Office, Viale Beatrice d'Este 37, 20122 Milan, ltaly; or call +39 02 58317318; or fax +39 02 58321266; or e-mail esolatin@tin.it; or see http://www.cancerworld.org; or e-mail afrasson@hotmail.com

Prostate cancer

September 1-3, 2002

The ESTRO teaching course "Brachytherapy for Prostate Cancer" will take place in Utrecht, the Netherlands.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Lung cancer

September 1-4, 2002

The "8th Central European Lung Cancer Conference" will be offered in Vienna, Austria.

Contact Conference Secretariat, Mondial Congress, Faulmanngasse 4, A-1040 Vienna, Austria; or call +43 1 588 04 0; or fax +43 1 586 91 85; or e-mail congress@mondial.at

Lung cancer

September5-7, 2002

The "2nd International Conference on New Perspectives in the Treatment of Small Cell Lung Cancer" will be offered in Lausanne, Switzerland.

Contact Imedex, 70 Technology Drive, Alpharetta, GA, 30005 3969 USA; or call +1 770 751 7332; or fax +1 770 751 7334; or e-mail meetings@imedex.com; or see http://www.imedex.com

Lung cancer

September 8-12, 2002

The "IASLC Workshop on Progress and Guidelines in the Management of Non Small Cell Cancer" will be offered in Bruges, Belgium.

Contact Secretariat, P. van Houtte, Dept. Radiotherapy, Institute Jules Bordet, Rue Heger-Bordet 1, B-1000 Brussels, Belgium; or call +32 2 541 3830; or fax +32 2 538 7542; or e-mail paul.vanhoutte@bordet.be

Medical physics

September 9-13, 2002

The "10th International Congress on Boron Neutron Capture Therapy" will take place in Essen, Germany.

Contact Dr. Ray Moss with e-mail moss@jrc.nl

Radiation therapy

September 17-21, 2002

The 21st Annual ESTRO Meeting will take place in Prague, Czech Republic.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Oncology

September 19-21, 2002

The ESO course "The Challenge of Cancer. A Central Role for General Practice" will take place in Dublin, Ireland.

Contact ESO Headquarters, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 02 43359611; or fax +39 02 43359640; or e-mail esomi@tin.it; or see http://www.cancerworld.org

Neuroradiology

September 16-28, 2002

The course "Johns Hopkins Neuroradiology Review" will take place in Baltimore, Maryland, USA.

Contact Conference Co-ordinator, Office of Continuing Medical Education, Johns Hopkins University School of Medicine, Turner 20/720 Rutland Avenue, Baltimore, Maryland 21205-2195, USA; or call +1 410 955 2959; or fax +1 410 955 0807; or e-mail cmenet@jhmi.edu; or see http://www.med.jhu.edu/cme

Ultrasound

September 20-22, 2002

The course "31st Annual Diagnostic Ultrasound in Gynecology and Obstetrics and Abdomen" will take place in Baltimore, Maryland, USA.

Contact Conference Co-ordinator, Office of Continuing Medical Education, Johns Hopkins University School of Medicine, Turner 20/720 Rutland Avenue, Baltimore, Maryland 21205-2195, USA; or call +1 410 955 2959; or fax +1 410 955 0807; or e-mail cmenet@jhmi.edu; or see http://www.med.jhu.edu/cme

Oncology

September 29 – October 3, 2002

The "2nd World Assembly on Tobacco Counters Health" will be offered in New Delhi, India.

Contact Convenor, WATCH 2002, 509-B, Sarita Vihar, New Delhi 110 044, India; or call +91 11 694 4551; or fax +91 11 694 4472; or e-mail cancerak@del6.vsnl.net.in; or see http://www.watch-2000.org

Radiation therapy

October 6-9, 2002

ASTRO Annual meeting will be held in New Orleans, Louisiana, USA.

Contact American Society for Therapeutic Radiology and Oncology Office, 1891 Preston White Drive, Reston, VA 20191, USA; or see http://www.astro.org

Cancer imaging

October 7-9, 2002

The 3rd Annual Teaching Course will be organised by International Cancer Imaging Society (ICIS 2002) and it will take place in Paris, France.

Contact ICIS Secretariat, BIR Conference Office, 36 Portland Place, London, W1B 1AT, U.K.; or call +44 20 7307 1416; or fax +44 20 7307 1414; or e-mail rebecca.gladdish@bir.org.uk

Salivary glands

October 7-12, 2002

The master course about cancer in salivary glands will take place at European Institute of Oncology in Milan, Italy.

Call P. Lonati, +39 02 5748 9490; or fax +39 02 5748 9491; or e-mail head&neck@ieo.it

Colorectal cancer

October 24-25, 2002

The "2nd Colorectal Cancer Conference" will take place in Rome, Italy.

Contact ESO Office, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 02 43359611; or fax +39 02 43359640; or e-mail esomi@tin.it; or see http://www.cancerworld.org

Radiation oncology

November 10-16, 2002

The ESTRO teaching course "Evidence-Based Radiation Oncology: Methodological Basis and Clinical Application" will take place in Tenerife, Spain.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Breast cancer

November 12-13, 2002

The ESO course will take place in New York, USA.

Contact ESO Headquarters, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 02 43359611; or fax +39 02 43359640; or e-mail esomi@tin.it; or see http://www.cancerworld.org; or R. Boschi-Belgin, ESO US Office, American-Italian Cancer Foundation, 112 East 71st Street – 2B, New York – NY 10021, USA; Phone +1 212 6289090; Fax +1 212 5176089; e-mail aicf@aicfonline.org; http://www.aicfonline.org

Breast cancer

November21-23, 2002

The ESO course "Current Breast Cancer Management" will take place in Johannesburg, South Africa.

Contact ESO Headquarters, Viale Beatrice d'Este 37, 20122 Milan, Italy; or call +39 02 43359611; or fax +39 02 43359640; or e-mail esomi@tin.it; or see http://www.cancerworld.org

Radiation oncology

March 15-19, 2003

The "2nd International Conference on Translation Research and Pre-Clinical Strategies in Radiation Oncology, ICTR 2003" will be offered in Lugano, Switzerland.

Fax +41 91 820 9044, or e-mail jbernier@pop.eunet.ch, or see http://www.osg.ch/ictr2003.html

Biomedicine

April 2-4, 2003

The "5th International Conference on Simulations in Biomedicine" will be offered in Ljubljana, Slovenia.

Contact Ms. Gabriella Cossutta, Conference Secretariat, Biomedicine 2003, Wessex Institute of Technology, Ashurst Lodge, Ashurst, Southampton, SO40 7AA, UK; or call +44 238 029 3232; or fax +44 238 029 2853; or e-mail gcossutta@wessex.ac.uk; or see http://www.wessex.ac.uk/conferences/2003/biomed03

Allergology and clinical immunology

June 7-11, 2003

The "22nd Congress of the European Academy of Allergology and Clinical Immunology" take place in Paris, France.

Contact Congrex Sweden AB, Attn: EAACI 2003, Linnegatan 89A, P.O. Box 5619, SE-114 86 Stockholm, Sweden, or call +46 8 459 66 00; or fax +46 8 661 91 25; or e-mail eaaci2003@congrex.se; or see http://www.eaaci.org

Lung cancer

August 10-14, 2003

The "10th World Conference of the International Association for the Study of Lung Cancer" will be offered in Vancouver, Canada.

Contact 10th World Conference of Lung Cancer, c/o International Conference Services, 604-850 West Hastings, Vancouver BC Canada V6C 1E1, or call +1 604 681 2153; or fax +1 604 681 1049; or e-mail conference@2003worldlungcancer.org

Radiation therapy

September 21-25, 2003

The ESTRO 22 / ECCO 12 Meeting will take place in Copenhagen, Denmark.

Contact FECS office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.fecs.be

Radiation therapy

October 19-23, 2003

ASTRO Annual meeting will be held in Salt Lake City, Utah, USA.

Contact American Society for Therapeutic Radiology and Oncology Office, 1891 Preston White Drive, Reston, VA 20191, USA; or see http://www.astro.org

Radiation therapy

September 12-16, 2004

The 23rd Annual ESTRO Meeting will be held.

Contact ESTRO office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.estro.be

Radiation therapy

October 3-7, 2004

ASTRO Annual meeting will be held in Atlanta, USA.

Contact American Society for Therapeutic Radiology and Oncology Office, 1891 Preston White Drive, Reston, VA 20191, USA; or see http:// www.astro.org

As a service to our readers, notices of meetings or courses will be inserted free of charge.

Please sent information to the Editorial office, Radiology and Oncology, Zaloška 2, SI-1000 Ljubljana, Slovenia.

- *v svetu največ predpisovani sistemski antimikotik*
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kriptokokni meningitis	*prvi dan 400 mg, nato od 200 do 400 mg na dan*
vzdrževalno zdravljenje	*200 mg na dan*

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Activity of "Dr. J. Cholewa" Foundation for Cancer Research and Education – A Report for the Second Quarter of 2002

The new circumstances, difficulties and problems associated with maintaining regular contacts with the donors were taken into consideration and seriously discussed on all levels by the members of the "Dr. J. Cholewa Foundation for Cancer Research and Education". It is hoped that some of the new approaches considered in contacts and communications with the donors will produce some tangible results in the near future. It is by now commonly understood that it would be naive to expect the same level of generosity by the donors in time of economic downturn following the unfortunate and violent events on September 11th, 2001, in New York and later also in the other parts of the world.

The decision was taken to increase the amount of the "Dr. J Cholewa Foundation for Cancer Research and Education" annual prize in order to give further incentive to young researchers in all parts of Slovenia. It is a long-time held position of the Foundation that high quality research work in oncology and related scientific fields is taking place and should be further encouraged in all parts of Slovenia where the interest to promote such research exists. It is thus perceived that the quality of research will improve and that the results of cancer research may find its way to the practical application in hospital wards a lot easier, and that in this way the attempts to publish and present the research results in respectable and influential international oncology journals, international meetings and conferences and other events of scientific importance, may gain another impetus. The Foundation therefore also continues to support the regular publication of "Radiology and Oncology" international scientific journal that is edited, published and printed in Ljubljana, Slovenia. With this in mind, a number of grants was thus also awarded to experts from various parts of Slovenia in order to attend various conferences and meetings in the field of oncology in Slovenia and around the world.

The Foundation is sad to announce that Mr. Metod Rotar, one of its founding members, passed away in May 2002. Mr. Metod Rotar had a successful career in government and in banking during his lifetime, and his rich experience and knowledge were instrumental in bringing about the idea of the "Dr. J. Cholewa Foundation for Cancer Research and Education", in maintaining the high spirits and zeal for activity among its members during the initial and most important passes of the fledgling Foundation, as well as later, when it had to adapt to the new circumstances. Mr. Metod Rotar will be greatly missed by the remaining members of the Foundation.

Tomaž Benulič, MD
Andrej Plesničar, MD
Borut Štabuc, MD, PhD

KEMOMED

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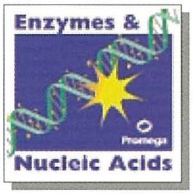

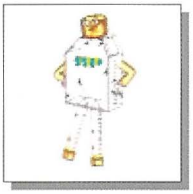

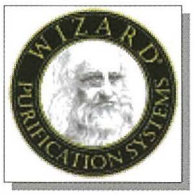

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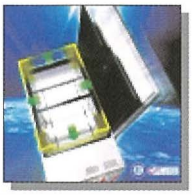

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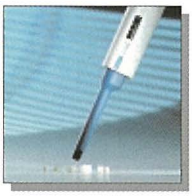

ELEKTRONSKE IN MEHANSKE AVTOMATSKE PIPETE

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zastopa naslednja podjetja

Köttermann (Nemčija):
laboratorijsko pohištvo, varnostne omare za kisline, luge, topila, pline in strupe, ventilacijska tehnika in digestorji

DAKO (Danska):
testi za aplikacijo v imunohistokemiji, patologiji, mikrobiologiji, virologiji, mono- in poliklonalna protitelesa

SVANOVA Biotech (Švedska):
Elisa testi za diagnostiko v veterini

NOVODIRECT BIOBLOCK (Francija):
kompletna oprema in pripomočki za delo v laboratoriju

GFL (Nemčija):
laboratorijski aparati, omare in skrinje za globoko zamrzovanje

ANGELANTONI SCIENTIFICA (Italija):
hladilna tehnika in aparati za laboratorije, transfuzijo, patologijo in sodno medicino

EHRET (Nemčija):
laminar flow tehnika, inkubatorji, sušilniki, suhi sterilizatorji in oprema za laboratorijsko vzrejo živali - kletke

INTEGRA BIOSCIENCES (Švica):
laboratorijska oprema za mikrobiologijo, biologijo celic, molekularno biologijo in biotehnologijo

CORNING (ZDA):
specialna laboratorijska plastika za aplikacijo v imunologiji, mikrobiologiji-virologiji, ipd., mehanske eno- in večkanalne pipete in nastavki

EVL (Nizozemska):
diagnostični testi za uporabo v veterinarski medicini

HÜRNER (Nemčija):
ventilacijska tehnika

CSL - Biosciences:
diagnostični testi za uporabo v veterinarski medicini

BIOMERICA (ZDA):
hitri testi za diagnostiko, EIA /RIA testi

CHARLES ISCHI (Švica):
specialna oprema za testiranje izdelkov v farmacevtski industriji;aparati za procesno kontrolo in kontrolo kvalitete

ROSYS - ANTHOS (Avstrija):
fotometri, avtomatski pralni sistem za mikrotitrine plošče

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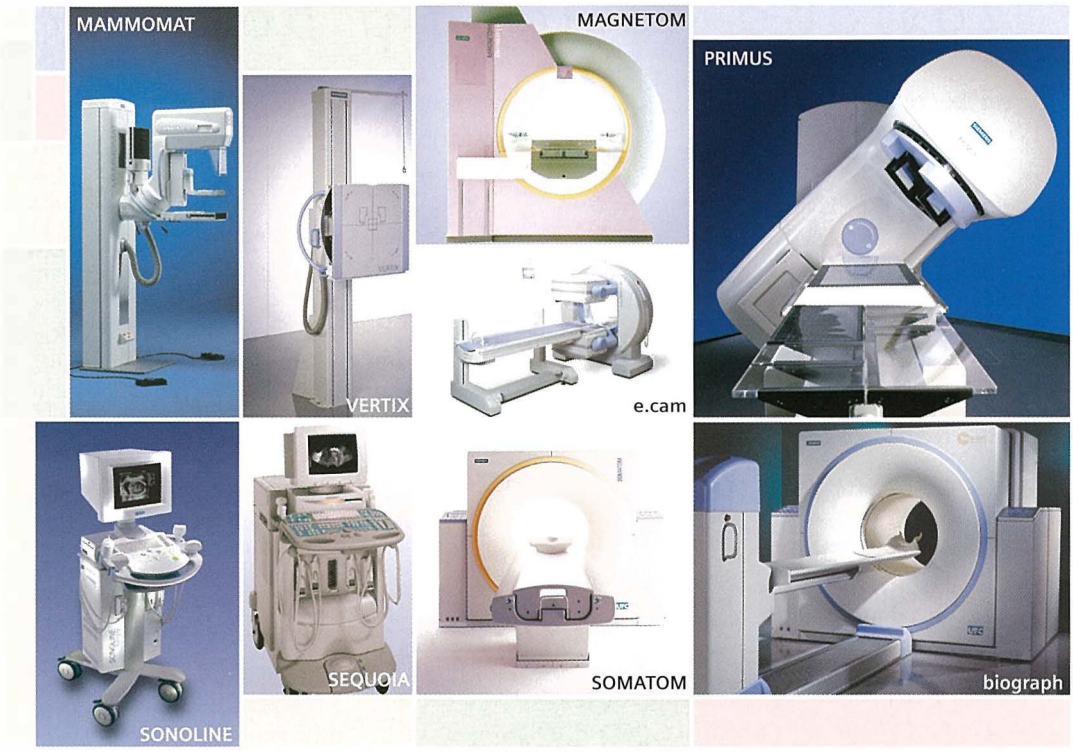

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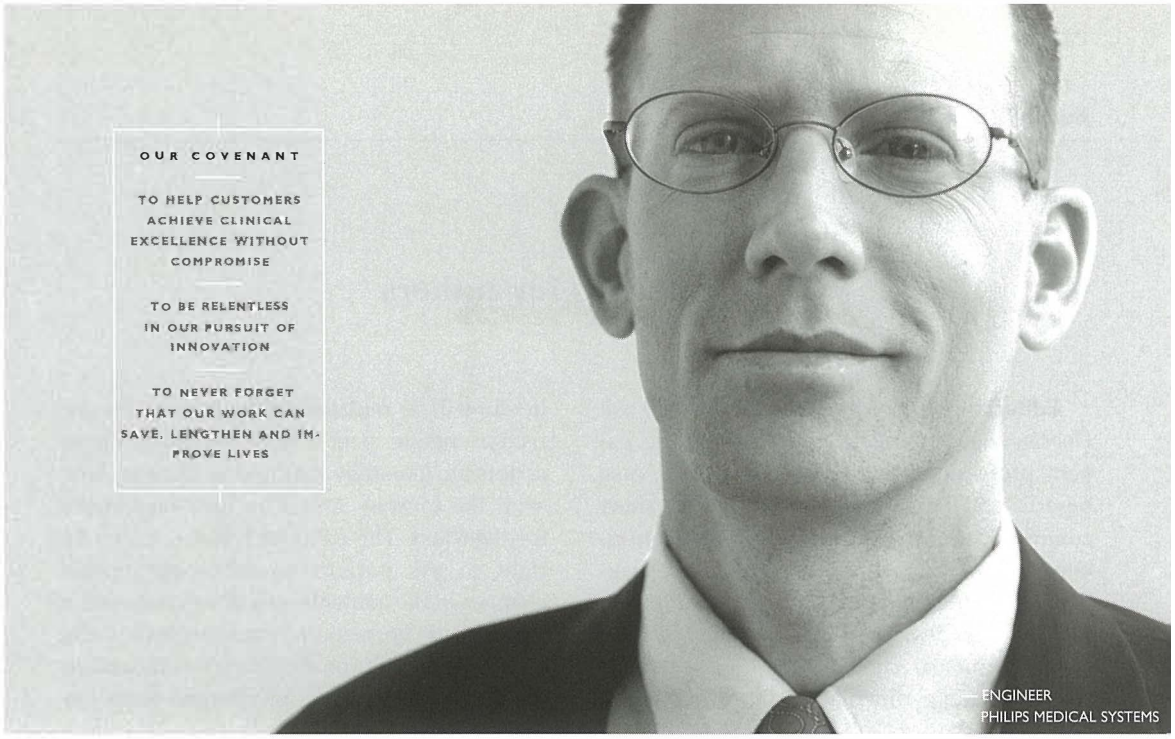

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Introduction should state the purpose of the article and summarize the rationale for the study or observation, citing only the essential references and stating the aim of the study.

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