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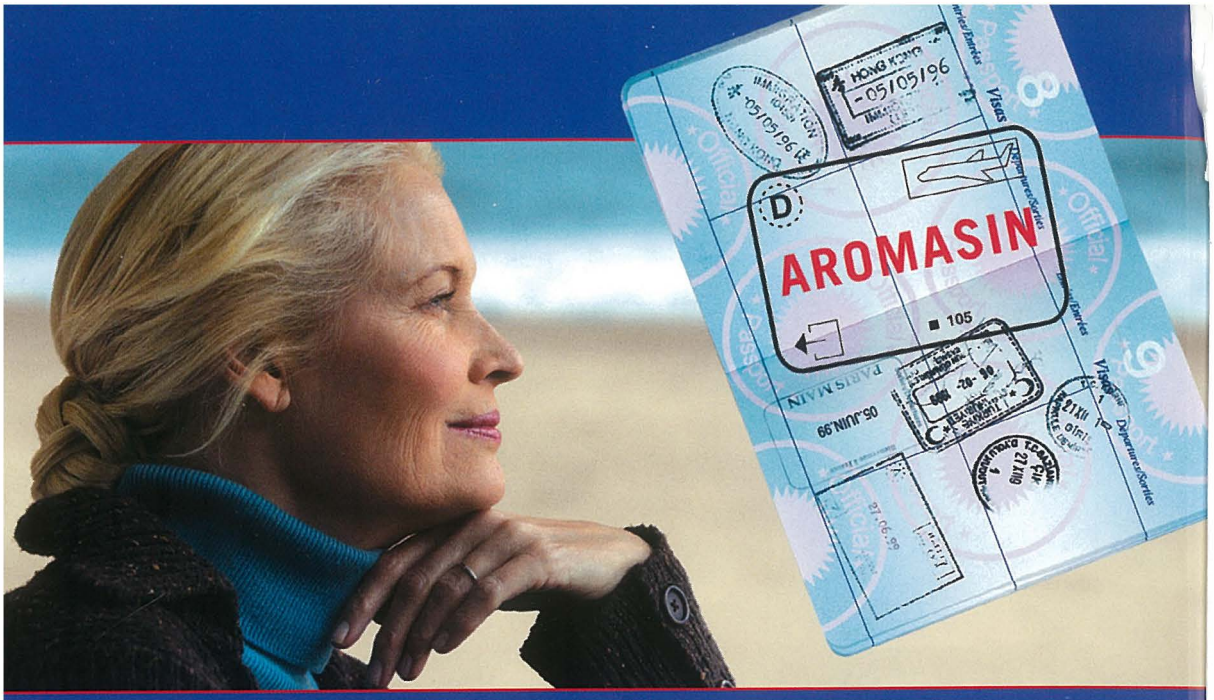

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CONTENTS

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Radiol Oncol 2006; 40(4): 205-9.

Intracapsular and para- articular chondroma of knee: a report of four cases and review of the literature

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University Clinic for Orthopaedic Surgery, Skopje, Macedonia

Background. *Intracapsular and para-articular chondroma is a rare variant of the extraskeletal chondromas. It arises from the capsule and/or the para-articular connective tissue of the large joints (mostly the knee) and is a result of cartilaginous metaplasia. In course of time these tumors ossify and this is where their second name comes from: Para-articular osteochondromas. According to Jaffe, not dependent on the degree of ossification of this tumor, there is one single entity in question.*

Cases report. *We report four new cases of para-articular chondroma of the knee. On physical examination there was slow-growing solid mass in the knee and moderate pain, the radiological findings and CT scan show soft-tissue mass with variable amount of ossification, and on histological examination the presence of mature hyaline and connective cartilage was confirmed in all of the cases.*

Conclusions. *The diagnosis of these benign tumors is made with correlation of clinical, radiological and histological features. The treatment of choice is surgical excision in toto.*

Key words: chondroma, osteochondroma, knee

Introduction

Extraskeletal chondromas are benign tumours which appear in three variants: synovial chondromatosis, para-articular chondroma and soft tissue chondroma. The first type is very common, but the last two variants are quite rare and they may show atypical features.[1-3]

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The fibrous coat of the capsule of a joint and/or the para-articular connective tissue, very rare, can suffer cartilaginous metaplasia. As an end result of this metaplasia, intracapsular or para-articular chondromas are formed. In time, they usually ossify so they are also known as capsular and para-articular osteochondromas. Mostly seen in the large joints (the knee), they vary in size depending on the size of the joint.[4,5] We have found only 30 cases of para-articular chondromas in the reviewed literature.[3,6,7] We report four new cases of capsular and para-articular chondroma of the knee with their clinical, radiological and histological features.

Case reports

Case 1

A male patient, 24, reports with painful mass on the medial side of the right knee, with no record of trauma. He first noticed it one year prior to the examination. On physical examination the timorous formation is movable, tender on palpation, produces pain during active motion. The profile X-ray shows para- and supra-patellar soft tissue tumorous formation (Figure 1a). On CT- scan this soft-tissue mass is clearly seen, oval shaped and intracapsular (Figure 1b). The tumour was surgically excised. During the operation, the intracapsular but extrasynovial position of the tumour was confirmed. It was oval shaped, 8 x 5 x 2.5 cm. The histological examination showed mature hyaline cartilage with foci of mixomatous tissue with benign characteristics. The diagnosis was: intracapsular chondroma without ossification.

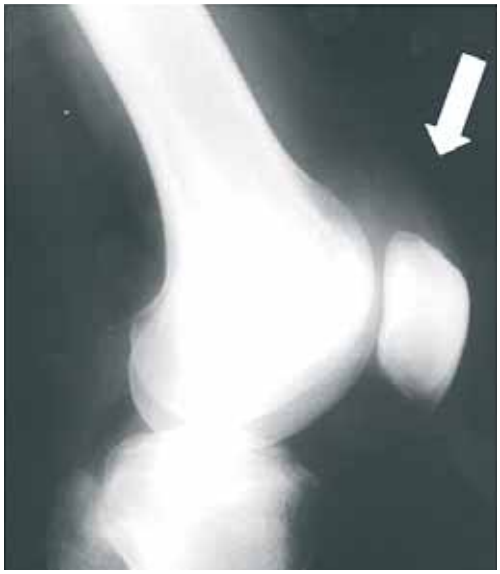

Figure 1a. *Case 1.* Lateral radiograph of the right knee: the arrow points to a parapatellar soft tissue mass.

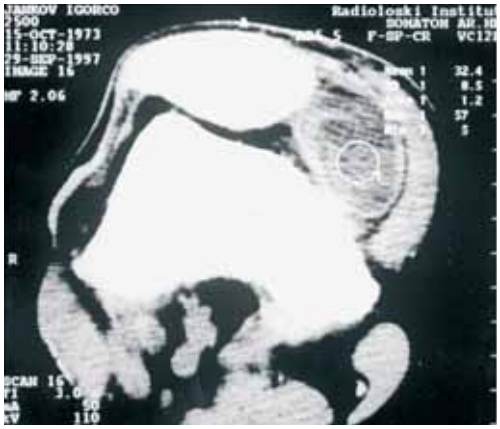

Figure 1b. *Case 1.* CT scan of the same knee*:* parapatelar, intracapsular soft tissue mass which has displased the patella.

Case 2

A female patient, age 41 complains of a solid mass on the lateral aspect of the left knee that has been slowly growing for the past two years. It caused limitation of joint movement and required surgical removal. The lateral radiograph of the left knee showed infrapatellar ossified mass (Figure 2a), whilst the CT- scan showed mostly ossified, encapsulated tumor just

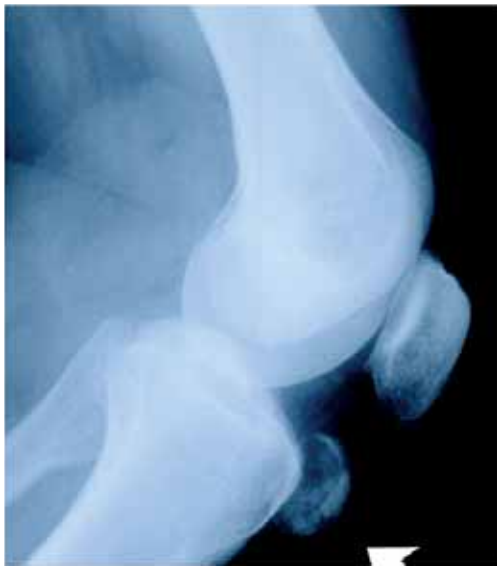

Figure 2a. *Case 2.* Lateral radiograph of the right knee: arge infrapatellar ossified mass.

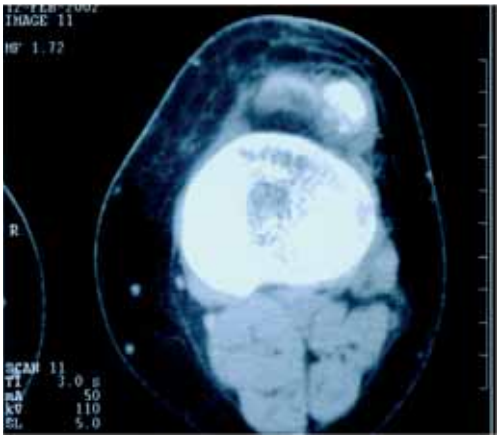

Figure 2b. *Case 2.* CT scan of the same knee: encapsulated ossified mass.

Figure 2c. *Case 2.* Macroscopic appereance of the excised tumour on cross-section.

beneath the lateral border of the patella, but not attached to it (Figure 2b). The surgically excised mass was oval, 3 x 3.5 x 2.5 cm (Figure 2c), situated in the continuity with the capsule of the joint, but extrasynovial. Macroscopically, on cross section there is a central zone of mature trabecular bone, surrounded by a hyaline cartilage cup (Figure 2c). On histological examination there was mature trabecular bone surrounded by hyaline cartilage with endochondral ossification. The diagnosis was: intracapsular chondroma with high rate of ossification.

Figure 3a. *Case 3.* Lateral X-ray of the knee shows the localization of the chondroma.

Case 3

A female patient aged 72 was admitted after a mild trauma of the left knee. Physical examination showed painful mass under the patella and limited flexion and extension of the knee which were present for more than 10 years. The recent trauma of the left knee caused pain and swelling of the knee. The lateral radiograph of the knee showed subpatellar, partly ossified mass (Figure 3a) and the transverse section on CT-Scan showed posttraumatic haematoma in the knee joint, as well as soft tissue tumour with ossification beneath the patella, situated in the para-articular connective tissue (Figure 3b). The diagnosis was: para-articular chondroma of the knee with ossification.

Case 4

A female patient aged 56 complained of intense pain and lack of extension in her right knee. There was no history of trauma, but she could remember heavy activities after which the progressive restriction of

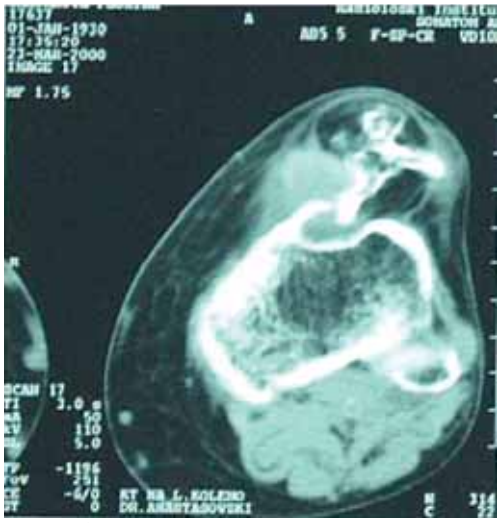

Figure 3b. *Case 3.* Transverse section on computer tomography of the knee shows proximal tibia, chondroma and posttraumatic haematoma (arrow).

the extension and pain started. History of the patient showed that she had slightly painful, slowly growing lump seated beneath and laterally of the patella for more than 30 years (Figure 4a). After the surgical extirpation of the para-articular chondroma (Figure 4b), full range of painless motion was regained.

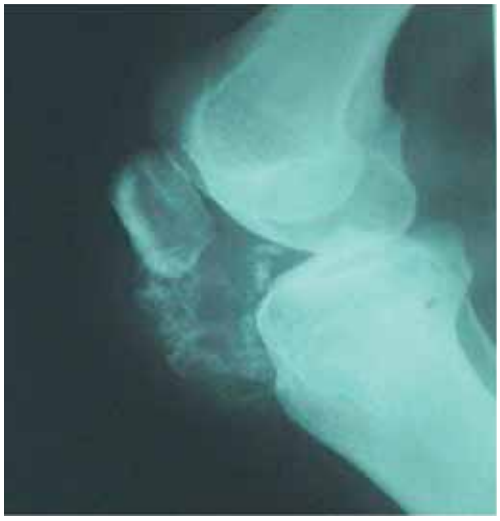

Figure 4a. *Case 4.* Lateral x-ray of the right knee, showing infrapatellar chondroma (arrow).

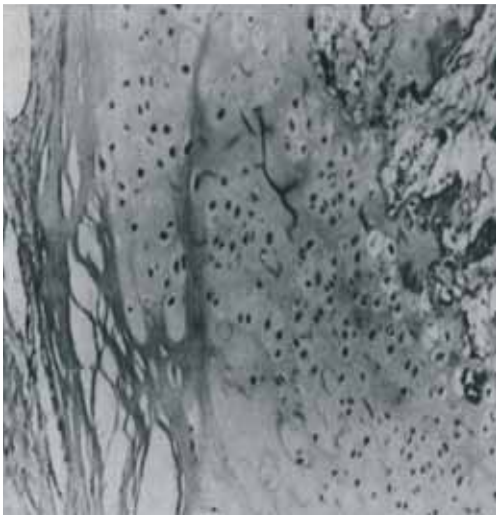

Figure 4b. *Case 4.* Histopathology of the chondroma (HE x600).

Discussion

Para-articular and intracapsular chondromas are rare benign tumours mostly seen in the vicinity of the large joints. They were often named capsular osteoma, osteochondroma or chondroma depending on the proportion of bone and cartilage.[8] According to Jaffe, there is only one single lesion in question regardless of the ossification, and in 1958 he classified all these terms under one entity: intracapsular and para-articular chondroma.[6] The Pathogenesis of these tumours is also controversial. They most likely originate from the connective tissue in the vicinity of the capsule of a joint or from the outer coat of the capsule as a result of cartilaginous metaplasia. Prior trauma is unlikely to play any significant role in the pathogenesis of these tumours. In the beginning comprising exclusively of cartilaginous tissue, in the course of time they usually ossify.[3,4] This is where their "second name" used in the literature comes from: osteochondromas.

From the relatively small number of reported cases we can conclude that, although

there have been cases in the ankle, elbow and the hip joint,[3,5] they are mostly seen in the knee joint.[4,7,8] The location is para-articular and intracapsular, mostly infrapatellar or medial to the patella. The reported age varies from 12 to 75 years. The clinical complaints are of some months to several years of local discomfort, moderate pain, slow growing mass and some degree of limited motion in the joint. Radiologically, there is a soft tissue mass with a different degree of central radiodensity due to ossification. Macroscopically their size varies, depending on the size of the involved joint, from 2 to 10 cm.

The four cases we report have all the features of the previously reported chondromas found in the literature. Clinically they present with moderate pain and restricted range of motion in the involved knee joint. On plain radiographs, there was a soft tissue mass with a different degree of ossification while the CT- scan has enabled us to make a more detailed analysis as to the exact position of the tumour (intracapsular or extracapsular), its relationship with the adjacent structures, its size and structure. In all of our cases, the tumours were intracapsular, but with no direct contact with the joint. Grossly, they were large, and the pathological analysis confirmed the presence of hyaline cartilage with variable amounts of mature trabecular bone. Using the definition of Jaffe, the diagnosis in all of the four cases was: para-articular chondroma. The treatment in all of the cases was surgical excision.

The diagnosis of these benign tumours is made clinically and radiologically in correlation with the pathological features. Although rarely seen, they should be considered in the differential diagnosis of soft tissue masses around the joints: haemathoma, bursitis, periosteal chondroma, synovial sarcoma, synovial chondrosarcoma. The treatment of choice for these tumours is surgical excision, while being careful not to injure the joint integrity. Malignant transformation has never been reported. With correct diagnosis unnecessary aggressive surgical treatment will be avoided.

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Radiol Oncol 2006; 40(4): 211-5.

case report

Doppler ultrasound in the diagnosis and follow-up of the muscle rupture and an arteriovenous fistula of the thigh in 12 year boy

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[2]*Department of Pediatric Radiology, Children's Hospital, Zagreb, Croatia*

Background. *With this case report the authors wish to present the accuracy of non-invasive vascular imaging methods, especially Doppler ultrasound, in the evaluation of the muscular trauma and periskeletal soft tissue vascular anomalies.*

Case report. *Twelve year-old boy has been admitted with the right femoral quadriceps muscle traumatic rupture. Postoperative B-mod sonography (US) visualised recidivuous haematoma and Power Doppler depicted hypervascularized area, suspected vascular malformation (angioma). Doppler findings obtained on the right thigh vasculature gave us reasons to think about posttraumatic arteriovenous fistula. Doppler has been repeated in the specialized paediatric institution with the same results. Digital subtraction angiography, 8 months after trauma, did not confirm suspicions reported in US findings. Spiral computed tomographic angiography (CTA) performed 11 months after trauma clearly depicted a lesion which had been repeatedly described in US findings. Fourteen months after trauma the vascular surgeon performed the deep femoral artery muscular branches ligation, but in the official report only arteriovenous fistula was mentioned. After the surgery the patient was clinically better. The aetiology of the right femoral arteriovenous fistula and hypervascularized structure remains unclear.*

Conclusions. *Every inadequately behaving, recidivous posttraumatic haematoma should be evaluated with Doppler ultrasound. CTA can be performed if it is needed to clarify US findings.*

Key words: hematoma – ultrasonography; arteriovenous fistula; muscle, skeletal - injuries

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Introduction

Soft tissue injury and its complications can be accurately evaluated with ultrasonography (US) because this method has possibilities of multiplanar approach, the dynamic examination of muscle during the contraction and rest and assessment of the potential concomitant vascular injury with Doppler modalities.[1-4] US doesn't carry

danger of ionizing radiation and it is widely available; these facts make US the imaging method of choice for the diagnostic evaluation of muscular injuries and first method for vascular injury diagnosing.

A muscle rupture of the lower limbs with consequent haematomas is often related with a sports injury. Haematoma is the most important sign of a muscle rupture, it is usually depicted as a hypo- or anechoic circumscribed lesion[1-3] an arteriovenous (AV) fistula is an abnormal communication between the arterial and venous systems. AV fistulas of the extremities are the consequence of trauma or medical procedures in most of the cases. Post-traumatic AV fistula is usually the consequence of penetrating trauma, very rarely after blunt trauma.[5-7]

Non-invasive imaging diagnostic methods, such as US, computed tomography (CT) and magnetic resonance imaging (MRI) have big potentials for the safe and even quick assessment of vascular anomalies and traumatic vascular lesions.[8] Doppler ultrasound methods can give the majority of necessary information about traumatic vascular lesions and vascular anomalies, especially if US is combined with another vascular imaging method, such as computed tomographic angiography (CTA) and magnetic resonance angiography (MRA).[3,4,9-12]

Case report

Twelve year-old boy was admitted with signs of the right femoral quadriceps muscle traumatic rupture caused by sudden extension during the football match, six months after trauma actually happened. B-mod US, performed before the surgical intervention showed the right femoral quadriceps muscle rupture with haematoma. First postoperative US included B-mod and Doppler modalities (colour and power Doppler). B-mod US depicted recidivous haematoma. With Doppler methods high flow and high systolic peak values were revealed in the popliteal, superficial and deep femoral vein, the AV communication was suspected (Figure 1)

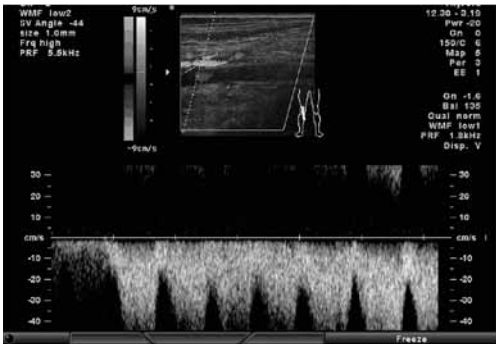

Figure 1. First Doppler ultrasound registered high flow and high systolic peak values in the popliteal, superfitial and deep femoral vein (horizontal orientation).

Hypervascularized area with sonographic characteristics of vascular malformation (haemangioma) was depicted near the haematoma, ventrolateraly in the proximal third of the thigh (Figure 2). Recidivous haematoma was evacuated by punction after the US control. Clinically, the patient had bigger diameter of all parts of the right leg and oedema ventrolateraly in the right femoral region, but without a thrill and bruit over the site of the muscle injury.

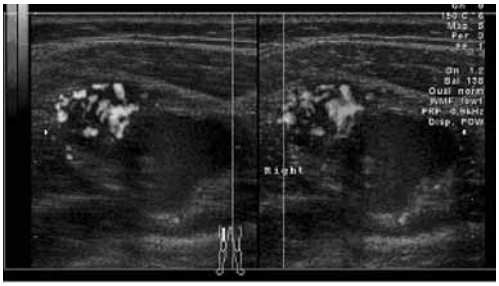

Figure 2. Hypervascularized area with sonographic characteristics of vascular malformation (hemangioma) positioned closely to the hematoma ventrolateraly in the proksimal third of the thigh (horizontal orientation).

The patient has been sent into the paediatric hospital to clarify the suspicion of the post-traumatic AV communication (fistula) and the right thigh vascular malformation. Control US and axial CT scan showed again recidivous haematoma and right femoral hypervascularized structure. The digital subtraction angiography (DSA) indicated by the paediatric surgeon and performed 8 months after trauma, also depicted neither AV fistula nor vascular malformation.

The repeated Doppler US, performed 10 and 11 months after trauma, showed a higher flow with high peak systolic values only in the deep femoral vein and the reduction of the right femoral hypervascularized structure size. The spiral CT scan, performed 11 months after trauma, depicted a hypervascularized lesion supplied from the deep femoral artery muscular branches positioned ventrolateraly in the right thigh proximal third. The hypervascularized lesion was equally good opacified with contrast material in the arterial and venous phase, with one avascular zone ventromedially (Figures 3, 4). The patient still had swollen right leg but there was no palpable mass in the area of vascular malformation described in the CT report with thrill and bruit over them.

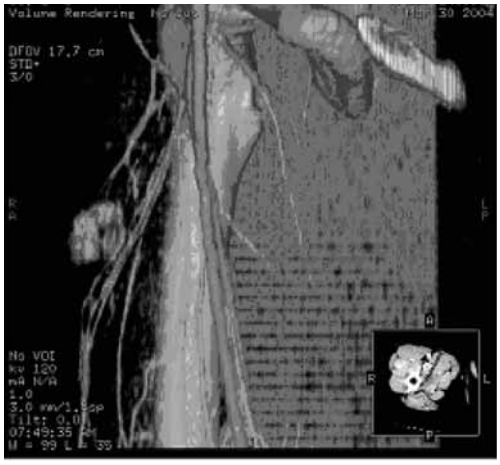

Figure 3a. Volume-rendering VR reformation – right femoral region-coronal plane (vertical orientation).

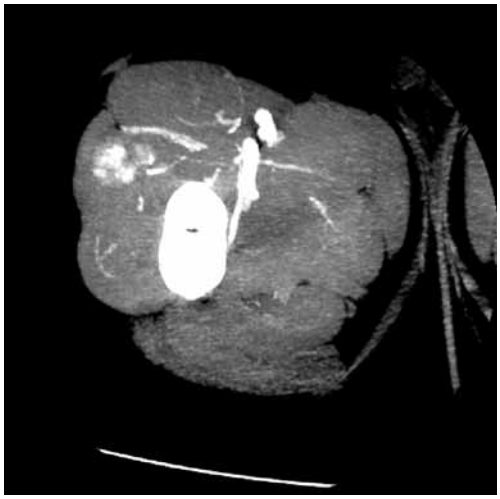

Figure 3b. Multiple intensity projection reformation MIP- right femoral region –axial plane (horizontal orientation).
Hypervascularized lesion located ventrolaterally in right thigh proximal third, equally good opacified with contrast material in the arterial and venous phase, with one avascular zone ventromedially. A lesion receives arterial supply from the deep femoral artery muscular branches.

The vascular surgeon performed the deep femoral artery muscular branches ligation, 14 months after trauma. In the surgery report only AV fistula was mentioned. After the surgery clinical manifestations and AV fistula ultrasonographic signs disappeared. This was an indirect confirmation of posttraumatic AV communication, which had not been supported with the digital subtraction angiography. The control US, performed two years after the surgical intervention showed neither haematoma nor AV communications, and the patient was clinically better without right leg oedema.

Discussion

Ultrasonography is a standard diagnostic method for the evaluation of soft-tissue structures trauma. Doppler ultrasound vas-

cular imaging is routinely included in the assessment of suspected vascular trauma. Duplex ultrasonography has sensitivity 95%, the specifity 99% and 98% accuracy in the assessment of peripheral vascular injuries, even 100% sensitivity and specifity compared with the conventional arteriography and operative exploration by Fry and colleagues 1994.[4] Doppler vascular imaging can help to detect the origin and pattern of vascular supply and the degree of blood flow in periskeletal soft tissue masses.[3,4] The combination of B-mod and Doppler sonography has 90% sensitivity and 91% specifitiy and 91% accuracy in the evaluation of musculoskeletal masses.[9] Soft tissue vascular masses can be distinguished with these characteristics: morphostructural features, the presence of colour or power signals, the site of vascular branches, their calibre and course, the number of afferent vascular poles, resistance index, vessel density and peak flow velocities.[10,11] Haemangioma and AV malformation have higher vessel density than other vascular malformations. There is no statistically significant difference between haemangioma and vascular malformation in vessel density and mean peak velocity. Solid-tissue mass is the factor for differentiation between haemangioma and vascular malformation.[11,12]

AV fistula clinical manifestations in the extremities usually are swelling of the injured limb, a thrill and bruit over the site of injury, but if the thrombus has occluded the AV communication the appearance of these signs will be delayed. The severity of AV fistula clinical manifestation can vary from local changes, as it was in this case, till the venous hypertension and congestive heart failure.[5,6]

In the case reported in this article the patient had unrecognised AV fistula. The penetrating injury of the right thigh was denied by the patient. There were no characteristic clinical signs like bruit and thrill over the region where the AV fistula was situated, although the right leg was swollen. The aetiology of the AV fistula and hypervascularized structure near the femoral quadriceps muscle rupture remained unclear. Working hypothesis about vascular malformation injured by trauma was not confirmed with DSA and operative findings. To the authors' knowledge the differential diagnosis of posttraumatic bleeding of a congenital AV-malformation has not yet been reported.

In this case of inadequately behaving posttraumatic haematoma, the correct diagnosis of an abnormal AV communication, an AV fistula, was made on the non-invasive vascular imaging methods findings (Doppler and CT angiography) ground. DSA didn't depict a right thigh AV fistula the existence of which was indirectly confirmed with the disappearance of clinical signs after the deep femoral artery muscular branche ligation, an AV fistula feeding artery. The point is that every inadequately behaving, recidivous posttraumatic haematoma should raise the suspicion of vascular injury, and must be evaluated with the vascular imaging methods. The facts presented in this case report support opinion that non-invasive vascular imaging methods like Doppler ultrasound and CT angiography can give enough information for diagnostic and therapeutic decisions and a follow-up after the treatment.

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Images in clinical medicine

Carcinoma of the lower lip

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In 71-year-old patient, the squamous cell carcinoma extended over 2/3 of the lower lip (Figures 1, 2). Surgical treatment would apparently be too mutilating; the patient was therefore referred to radiotherapy and received tmour dose (TD) 40 Gy in 10 fractions in 2 weeks, ortovoltage machine (Pantak).
The tumour regressed completely. A photo of the patient was taken two years after the completed radiotherapy. On the last follow-up control ten years after the completed radiotherapy, no evidence of local or regional recurrence of the carcinoma was observed.

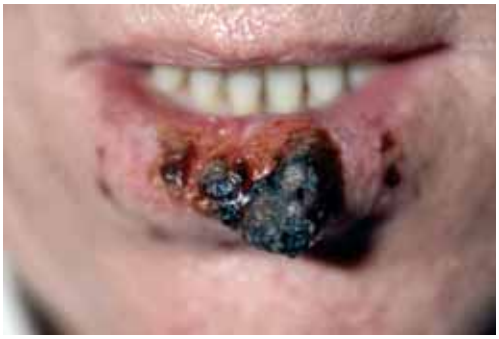

Figure 1. Huge carcinoma of the lower lip.

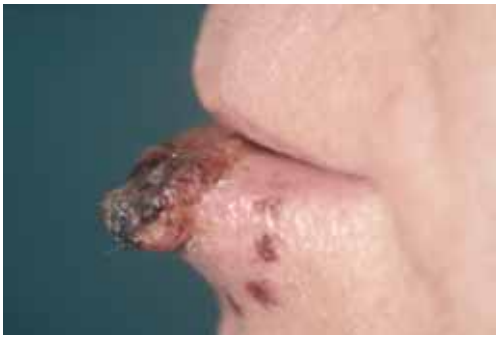

Figure 2. Exofitic huge carcinoma of the lower lip.

Figure 3. Lower lip, two years after the completed radiotherapy.

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review

Tumor vaccines

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Tumor vaccines have several potential advantages over standard anticancer regiments. They represent highly specific anticancer therapy. Inducing tumor-specific memory T-lymphocytes, they have potential for long-lived antitumor effects. However, clinical trials, in which cancer patients were vaccinated with tumor vaccines, have been so far mainly disappointing. There are many reasons for the inefficiency of tumor vaccines. Most cancer antigens are normal self-molecules to which immune tolerance exists. That is why the population of tumor-specific lymphocytes is represented by a small number of low-affinity T-lymphocytes that induce weak antitumor immune response. Simultaneously, tumors evolve many mechanisms to actively evade immune system, what makes them poorly immunogenic or even tolerogenic. Novel immunotherapeutic strategies are directed toward breaking immune tolerance to tumor antigens, enhancing immunogenicity of tumor vaccines and overcoming mechanisms of tumor escape. There are several approaches, unfortunately, all of them still far away from an ideal tumor vaccine that would reject a tumor. Difficulties in the activation of antitumor immune response by tumor vaccines have led to the development of alternative immunotherapeutic strategies that directly focus on effector mechanisms of immune system (adoptive tumor-specific T-lymphocyte transfer and tumor specific monoclonal antibodies).

Key words: cancer vaccines; antigens, neoplasms; immunotherapy

Introduction

Development of tumor vaccines is based on the researches that have shown that many tumors express tumor antigens and are able to elicit tumor-specific B- and T-lymphocyte responses. Tumor vaccines have several potential advantages over standard anticancer regimens. They are directed against tumor antigens and represent highly specific anticancer therapy. Inducing tumor-specific memory T-lymphocytes, they have potential for long-lived antitumor effects. Side effects of tumor vaccines are rare, in most cases limited to local reactions with minimal systemic toxicity (transient elevated body temperature, flu-like symptoms). Autoimmune reactions are also rare (vitiligo with melanoma vaccines).

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Tumor antigens

Genetic and epigenetic changes characteristic of carcinogenesis make cancer cells antigenically distinct from normal human cells.[1] Cancer cells express tumor-specific antigens and tumor-associated antigens. *Tumor-specific antigens* are ideal targets for antitumor therapy. They are protein products of mutated normal cell genes and are expressed only by cancer cells. They are foreign to immune system and therefore elicit high-affinity antitumor T-lymphocyte responses with low probability of intercurrent autoimmune reactions.[1-4] Their main disadvantages are that they are highly heterogenic and expressed only by certain types of tumors, therefore they cannot be used as a universal antigen in a cancer vaccine. A special subgroup of tumor-specific antigens are idiotypic sequences of B-cell membrane immunoglobulins or T-cell receptor.[5,6]

Tumor-associated antigens. Most antigens expressed by tumor cells are normal, nonmutated self-molecules to which immune tolerance exists.[3,7] There are several classes of tumor-associated antigens: *tissue-specific antigens* (PSA, melanocyte antigens), *oncofetal proteins* (normally expressed only during fetal development, foreign to immune system, reactivated in undifferentiated tumors), *cancer testes antigens* (normal testicular proteins, overexpressed on cancer cells, foreign to immune system - spermatocytes do not express MHC molecules), *overexpressed normal cell proteins* (HER2 Neu in breast carcinoma) and self-proteins with abnormal *posttranslational modifications* (overglycosilated mucins, as MUC1 in breast carcinoma, changes in glycosilation can expose kryptotopes, foreign to immune system).[1,9,10]

Viral oncoproteins (human papilloma virus proteins E6 and E7) are a special group of tumor antigens, playing a critical role in malignant transformation of infected cells. Being foreign to a body, they can induce high-affinity T-lymphocyte responses.[11,12]

Antitumor immune response

Tumor-specific cytotoxic T-lymphocytes are central effector cells of antitumor immune response. They are the only cells capable of efficiently killing cancer cells, inducing their apoptosis or lysing them by action of perforines and granyzimes. They are produced in cross-priming of naive CD8+ T-lymphocytes, mediated by mature dendritic cells (DC). The process is essential for induction of antitumor immune response and involves cross-presentation of antigenic peptides originating from extracellular proteins through MHC I molecules on the surface of DC to naive CD8+ T-lymphocyte.[9,13] The pathway involves endosome to cytosol shuffling of antigenic peptides from extracellular proteins mediated by TAP transporters, and is a major pathway of cross-presentation under physiological conditions.

Efficient cross-activation of naive CD8+ T-lymphocytes requires 3 types of cells, mature DC, naive CD8+ T-lymphocyte and helper T-cell, and 2 signals. S*ignal 1 (specific antigenic signal)* arises from interaction between antigenic peptide, presenting on dendritic cell MHC I molecule, and antigen specific T-cell receptor (TCR) of naive CD8+ T-lymphocyte; *signal 2 (costimulatory signal)* is mediated by costimulatory molecule B7 on the surface of DC and its CD28 receptor on naive CD8+ T-lymphocyte (two signal hypothesis).[9,13] For activation of naive CD8+ T-lymphocyte to be effective, licensing of DC is necessary. It is mediated by an interaction between CD40 ligand (CD40L) of DC and its receptor CD40 of helper T-cell, specific for antigenic peptide presented on MHC II molecule of DC. Interaction CD40 - CD40L results in an upregulation of cos-

timulatory molecules B7 on the surface of DC - DC licensing. B7 interact with CD28 receptor, providing a dominant costimulatory signal for the activation of naive CD8+ T-lymphocyte.

Immune tolerance for tumor antigens

Existence of tumor-specific lymphocytes and antitumor antibodies before and after vaccination has been found in many cancer patients; however, there has been no correlation with clinical improvement so far.[1] There are many reasons for inefficiency of tumor vaccines. Tumor vaccines contain mainly weakly immunogenic tumor-associated (self) antigens and elicit weak antitumor immune responses. Frequency of tumor-specific lymphocyte precursors, that arise during tumor vaccination, is small (≤1%) compared to the frequency of lymphocyte precursors (≥10%) against the infectious agents arising during classic vaccination.[8] Even more importantly, the population of potentially tumor-reactive lymphocytes is represented by low-affinity T-lymphocytes, as high-affinity self-reactive T-lymphocytes have been deleted in a process of self-tolerance.[8] Self-tolerance protects the body from autoimmune reactions and plays an essential role in inefficiency of tumor vaccines.[1]

Mechanisms of tumor escape

Tumors evolve many mechanisms to evade actively or silence antitumor immune response, what makes them poorly immunogenic or even tolerogenic.[14]

Tumor cells inefficiently present antigens to effector T-lymphocytes

Genetic instability of tumors results in changing the tumor antigenic profile.[15] Mutations of immunodominant tumor epitopes can prevent the recognition of a tumor cell by immune system.[1] Level of tumor peptide presentation through MHC I molecules can be so low that the tumors remain undetected by specific T-lymphocytes.[8] Mechanisms of antigen presentation and processing are defective in many tumors. Low levels of surface MHC I molecules are characteristic of many tumors and correlate with worse prognosis. However, total absence of surface MHC I molecules makes a tumor cell more susceptible for lysis by natural killer cells.[14]

Induction of tolerance - anergy or deletion- of tumor specific lymphocytes

Tumor cells actively participate in the induction of immune tolerance of tumor-specific lymphocytes. They interfere with maturation of DC, express surface Fas ligand inducing apoptosis of Fas positive tumor-specific lymphocytes, produce immunosuppressive cytokines (IL-10, TGF-β) and redirect immune response in the development of regulatory CD4+CD25+ T-lymphocytes that inhibit the action of effector T-lymphocytes.[1,13-17]

Tumor interference with function of dendritic cell

It has been found that DC are numerically and functionally defective in cancer patients.[9] Adoptively transferred tumor-specific T-lymphocytes in mouse tumor model become anergic soon after their transfer to a mouse that has already developed the tumor.[16] Anergy is caused by interaction of unmature DC, lacking a costimulatory signal, with tumor-specific T-lymphocytes. Level of DC maturation is essential in directing immune response either in antitumor immunity or in unresponsiveness to tumor antigens.[9] Mechanisms of tumor

cell interference with the function of DC involve an early and a late inhibition of DC maturation.[13] The early inhibition of DC maturation is a result of cytokine mediated redirection of granulo/monocyte precursors from DC line to monocyte/macrophage line, decreasing the number of circulating DCs and increasing the number of circulating monocytes/macrophages which are inefficient antigen-presenting cells in antitumor immunity.[13] The late inhibition of DC maturation is a result of tumor mediated suppression of antigen cross-presentation and DC costimulatory molecules expression. Many tumors downregulate the expression of heat shock proteins that participate in endosome to cytosol shuffling pathway and provide maturation signals for DC. IL-10 limits availability of lysosomal proteases that are essential for the production of antigenic peptides.[13] Phagocytosis of early apoptotic melanoma cells, rich in IL-10, inhibits the induction of DC costimulatory molecules.[13]

Peripheral deletion of tumor-infiltrating lymphocytes

Peripheral deletion of tumor-infiltrating lymphocytes is mediated by Fas ligand on the tumor cells inducing apoptosis of Fas receptor positive tumor-specific T-lymphocytes.[8] Expression of Fas ligand on esophageal carcinoma cells is an early sign of disease progression.[14]

Immunoregulatory CD4+CD25+ T-lymphocytes

Immunoregulatory CD4+CD25+ T-lymphocytes are important negative regulators of immune response and represent 5 to 10% of peripheral T-lymphocytes. They induce anergy of high-affinity self-reactive T-lymphocytes that have escaped central deletion process, and protect the body from autoimmune diseases and overdriven normal immune responses against microbes.[8,18] An increasing number of evidence show that they significantly suppress the antitumor immune response and participate in the induction of immune tolerance to tumor antigenes.[5,19] Simultaneous use of anti-CD25 monoclonal antibodies and tumor vaccine in a mouse tumor model increases the efficiency of the vaccine and prolongs the survival of the experimental animal. The ratio of peripheral immunoregulatory CD4+CD25+ T-lymphocytes correlates negatively with the prognosis of gastrointestinal malignancies.[23] Cancer patients (23 ± 4%) have increased numbers of peripheral immunoregulatory CD4+CD25+ T-lymphocytes compared to healthy (6 ± 3%) volunteers.[5,19]

Immunoregulatory control points

The high-affinity inhibitory CTLA-4 receptor expressed on activated T-lymphocytes competes with the lower-affinity stimulatory receptor CD28 for binding the B7 costimulatory molecules on DC. It is implicated in the induction of self-tolerance and regulates the amplitude of normal T-lymphocyte responses. Similar actions are mediated by the B7-H1 and B7-H4 molecules that are frequently over-expressed in pancreatic carcinoma. Monoclonal antibodies directed against the B7-H1 and B7-H4 molecules are already in development.[17]

Production of immunosuppressive cytokines and cytokine immunostimulation of tumors

Many cytokines (especially IL-10 and TGFβ), produced by tumor cells or tumor-infiltrating lymphocytes, have several different immunosuppressive actions.[14]

Cytokines can also accelerate tumor growth. The immunostimulatory actions of cytokines are seen mainly in hematologic

malignancies. However, most solid tumors express the low-affinity IL-2 receptor βμ (IL2R βμ) which correlates with the increased therapeutic resistance of a tumor. Cytokines should therefore be used cautiously in cancer patients as they could have detrimental effects on the survival of patients.

Tumor microenvironment

Infiltration of a tumor by tumor-specific lymphocytes is highly dependent on local tumor microenvironment.[20] Tumor microvasculature represents significant barrier for lymphocytes. Although peritumor regions are rich in lymphocytes and tumors are usually well vascularized, lymphocytic infiltration of tumor remains poor.[20] High-endothelial venules with activated endothelium that are important for entrance of lymphocytes in an inflamed tissues are rarely present in intratumor regions.[20] Tumor cells actively suppress expression of endothelial adhesion molecules by local secretion of angiogenic factors and cytokines. Poor lymphocyte infiltration is characteristic of many tumors and bears poor prognosis.

Tumor vaccines

Ideal tumor vaccine is a specific-tumor antigen expressed only by tumor cells (cannot induce autoimmune reactions) that participates in carcinogenesis and is crucial to tumor cell survival (preventing selection of immunoresistant clones during immunotherapy). It must be expressed in high levels at all stages of the disease and must be common (universal) to different tumors. It is foreign to immune system and elicits high-affinity cellular and humoral immune responses with long-lived antitumor immunological memory.[5,9]

The real situation is far from being ideal. Most tumor antigens are self-molecules tolerated by immune system.[7,21] Tumor-specific lymphocytes isolated from cancer patients are rare and mainly anergic. Antitumor immune response imposes selective pressure over a genetically unstable tumor and accelerates the emergence of immunoresistant clones. Simultaneously, the tumor develops many strategies to evade successfully antitumor immune response.[16,21] Novel immunotherapeutic strategies are therefore directed toward breaking the immune tolerance to tumor antigens, enhancing the immunogenicity of tumor vaccines and overcoming the mechanisms of tumor escape.[16,22] There are several different immunotherapeutic approaches, all of them unfortunately still far away from an ideal tumor vaccine that would reject a tumor.

The present role of tumor vaccines in cancer therapy is minor. They are used mainly as adjuvant treatment in the patients with advanced cancer. However, best results with tumor vaccines could be expected in a state of minimal residual disease after the majority of tumor burden has been removed by surgery or chemotherapy as the probability of immunoselection of resistant clones is the smallest and the immunosuppressive effects of tumor are least pronounced.

Cellular vaccines

Cellular vaccines are either autologous or allogenic and contain tumor cells or their lysates.

Classic autologous cellular vaccines contain attenuated patients' own tumor cells (requiring surgical resection of a sample of the patient tumor). Their primary advantages are that they contain all antigens of the patient's tumor, can be specifically tailored for each patient and in every moment match the tumor's changing antigenic profile.[23,24] Previous identification of tumor antigens is not required and there are no

limitations concerning the patient's HLA haplotype.[15,23] They induce polyclonal antitumor immune response that more readily overcomes several tumor evading strategies and imposes smaller immunoselective pressure over the tumor.[15] However, as the identity of tumor antigens is unknown, there are difficulties with the standardization of vaccine production protocol and measurement of postvaccination immune responses. Besides, variable immunogenicity of tumor antigens among different patients influences the efficiency of immune response elicited by vaccine.[15,24,25]

Allogenic cellular vaccines are based on the idea that tumors of the same type from different patients share many common antigens. They are prepared from cultured tumor cell lines, standardized, readily available and can be applied to many patients.[15,25] Canvaxin and Melacin are allogenic cellular vaccines, approved in adjuvant therapy of metastatic melanoma, and induce regression of melanoma lesions in 5 to 10% of treated patients.[22,25] The main disadvantage of cellular vaccines is their weak immunogenicity that can be improved by transfection of tumor cells with the genes that code for different immunostimulatory molecules (cytokines, chemokines, adhesion, MHC and costimulatory molecules) or by hybridizing tumor cells and DC.[15,16,25] Genetic modifications have been shown to increase importantly immunogenicity of tumor vaccines, however clinical improvement has remained poor. [16]

Peptide vaccines

Peptide vaccines are intended to stimulate T-lymphocyte responses to tumor-specific antigenic peptides presented on the surface of tumor cells through MHC I molecules. Namely, most tumor antigens originate in tumor cell cytosol or cellular organelles and present themselves in the complex with the surface MHC I molecules. Peptide vaccines have several advantages; they are easily produced, inexpensive, safe, synthesized in big quantities and represent a standardized, well defined antigen, allowing postvaccination immune response monitoring.[2,23] Their main disadvantage is MHC I allotype restriction that makes them useful only in the patients matching MHC I allotype.[2,23,27] Vaccination of cancer patients with one or two antigenic peptides has so far induced specific immune response in as many as 80% of patients; however, clinical improvement has been found only in 10 to 20% of patients.[2] It has been proposed that a combination of many peptides would be necessary to achieve clinical results.

Dendritic cell vaccines

DCs are professional antigen-presenting cells essential in cross-presentation and differentiation of naive tumor-specific CD8+ T-lymphocytes in efficient cytotoxic cells. The basis for the development of DC vaccines has been established with the protocols for *ex vivo* preparation of DC.[28] DC can be prepared from CD34+ precursor cells isolated from bone marrow or peripheral blood after their incubation with different cytokine combinations, as are TNFα, GM-CSF, Flt3 ligand, CD40 ligand and TGFβ.[28] Mature low-phagocytic DC are produced, expressing high levels of membrane costimulatory molecules. Alternatively, DC are prepared from peripheral blood monocytes in culture with GM-CSF and IL-4. These DC are unmature, highly phagocytic and efficiently take up tumor antigens (tumor cells, peptides, proteins, tumor exosomes, heat shock proteins) they are incubated with.[15,16,28] Another possibility of DC antigen loading is transfection of DC with cDNA or mRNA, coding for tumor antigens, mediated by viral vectors, electroporation or lipofection.[16,28] Still better method

is the transfection of DC with total tumor mRNA. After antigen loading of unmature DC is finished, it is necessary to induce DC maturation, mainly by TNFα, Toll-like receptor agonists (CpG oligonucleotides), IL-1β or IL-6.[16,21]

Tumor heat shock protein vaccines

An increasing number of evidence show that heat shock proteins (HSP), as are GP96 in HSP70 isolated from tumor cells, can induce a specific antitumor immune response. HSP are able to bind the antigenic peptides arising in a tumor cell, to be actively taken up by DC in a process of receptor mediated endocytosis and to induce DC maturation through the interaction with DC Toll-like receptors.[23,29] After HSP internalization, the antigenic peptides are released from HSP and enter antigen processing and cross-presentation process, finally emerging as a complex with MHC I on the surface of APC. There are many advantages of HSP vaccines. They contain many, if not all tumor antigenic peptides, induce polyclonal antitumor immune response, bring antigens to DC and induce their maturation.[23,29,30] The disadvantages of HSP vaccines are time consuming isolation of peptide-HSP complexes and unknown antigenic profile.[23,29] Many animal models have confirmed *in vivo* immunogenicity of HSP70 or GP96 vaccines. They have proven efficient in the induction of prophilactic and therapeutic antitumor immune responses in many preclinical trials and are now being tested in first- and second-phase clinical trials.[33] In a study by Castelli *et al*., the vaccination of colorectal carcinoma and melanoma patients by GP96 induced statistically significant antitumor T-cell immune response in 59% of melanoma patients and 47.8% colorectal carcinoma patients. A complete regression of melanoma lesions was achieved in 18% of patients and the survival of colorectal carcinoma patients was prolonged.[29]

Nanovesicular vaccines - tumor exosomes

Exosomes (nanovesicles) are small membranous vesicles, originating from late endosome. They bud from the membrane of subcellular multivesicular bodies, fuse with plasmalema and are released extracellularly, where they refuse with the neighbor cells' membranes. Exosomes are composed of different cytosolic and membrane proteins and have dual function. They represent a vehicle for removing redundant cellular proteins and are a pathway for trafficking proteins between cells, thereby participating in a complex intercellular communication.[31] The DC exosomes are enriched in adhesion proteins, costimulatory molecules, MHC I and MHC II molecules together with antigenic peptides - they have immunomodulatory capacity. The tumor cell exosomes are enriched in native tumor proteins and are constitutively secreted by tumor cells. They bring tumor antigens to DC and, through action of surface HSP70, accelerate self-internalization in DC. The incubation of DC with the tumor exosomes *in vitro* and *in vivo* in mouse tumor models results in the activation of specific cytotoxic T-lymphocytes. Because of important role in antitumor immune response and proven preclinical antitumor efficiency, the DC and tumor cell exosomes are being tested in the first-phase clinical trials.[31]

Idiotypic vaccines

Idiotypic vaccines use the variable region of B-lymphocyte membrane immunoglobulin as an antigen. The variable region contains epitopes unique to malignant B-lymphocyte clone and is therefore highly specific for the tumor.[6] Idiotypic epitopes elicit polyclonal immune responses. Multiple myeloma patients have antiidiotypic antibodies and

idiotype-specific T-lymphocytes in their blood. *In vitro* experiments and animal tumor models have shown that antiidiotypic immune response can destroy malignant myeloma cells.[5] Polyclonal nature of antiidiotypic immune response strongly reduces immunoselective pressure and the resultant emergency of immunoresistant myeloma cells. Idiotypic vaccines could therefore represent a promising immunotherapeutic antitumor strategy.[22] Their main disadvantage is their weak immunogenicity as idiotype is a self-protein. It has been shown that the conjugation of an idiotype with a strongly immunogenic adjuvant is necessary for eliciting an efficient antiidiotypic immune response.[23] Specific idiotypic protein can be produced from hybridoma cells or can be synthetisized by methods of recombinant gene technology. Total idiotypic protein can be produced, or better, only its single-chain variable fragment avoiding harmful reactions against the immunoglobulin constant region.[23] Lately, idiotype fusion DNA vaccines have emerged. They are composed of cDNA coding for heavy or light chain variable region linked to bacterial DNA or cDNA coding for the tetanus toxoid C fragment.[5]

Viral vaccines

Cervical carcinoma is caused by persistent infection of cervical epithelia with cancer-associated types of human papilloma virus (HPV). The genome of cancer-associated HPV is found in 99% of cervical malignant lesions.[12] HPV infects the basal cells of cervical squamous epithelia. HPV prolipheration and assembly are intimately linked to epithelial cell differentiation program; infective virions are produced only in fully differentiated epithelial cells.[12] As tissue damage with HPV infection is minimal and double helical RNA, an effective APC activator, is not produced during HPV cycle, the spontaneous anti-HPV immune response is weak.[11] Despite that, most HPV infections spontaneously disappear in few years.[11,12] As cervical carcinoma is caused by HPV16 or HPV18 in two thirds of patients, prophylactic and therapeutic vaccines are directed mainly against their antigens. Prophylactic vaccines contain recombinantly produced HPV16/HPV18 capside antigens L1, forming virus-like particles, and are entering in clinical use.[12] Two important randomized placebo controlled studies that included young sexually active women have shown the vaccination with HPV16/HPV18-like particles to be safe and effective and protects against persistent HPV infection and development of precancerous cervical lesions.[11] Therapeutic HPV vaccines are directed against HPV proteins E6 and E7 and are mostly experimental with limited clinical efficiency.[11,12]

Difficulties in activation of antitumor immune response by tumor vaccines have led lately to the development of alternative immunotherapeutic strategies directly focusing on the effector mechanisms of immune system. Such approaches include adoptive tumor-specific T-lymphocyte transfer and tumor-specific monoclonal antibodies.

Adoptive T cell transfer

Autologous tumor-specific T-lymphocytes isolated from the patient's peripheral blood, tumor or tumor-infiltrated lymph nodes are activated and multiplied *ex vivo* in the presence of specific T-cell epitopes and then returned to the patient.[16,32] There are several advantages of the adoptive T-cell transfer. It provides large numbers of tumor specific T-lymphocytes which are activated in the absence of inhibitory and tolerogenic tumor actions.[7] Compared to tumor vaccines, it is a better immunotherapeutic option for the patients with widespread disease and

high tumor burden.[32] However, the identity of tumor antigens used in the *ex vivo* activation of antitumor T-lymphocytes has to be known and the process of antigen identification is difficult and time consuming. Main disadvantages are decreased ability of transferred lymphocytes for tumor infiltration and their shorter survival that can be partially improved by adding IL-2.[16,32] Lymphodepletion with the resultant removal of regulatory T-lymphocytes, preceding adoptive T-cell transfer, is an important factor in achieving an efficient antitumor immune response mediated by the transferred tumor-specific lymphocytes.[7,21] Adoptive transfer of Melan-A/MART1 epitope-specific T-lymphocytes or GP100-specific CD8+ T-lymphocytes induced regression of melanoma lesions in metastatic melanoma patients; however, target melanoma epitopes were eventually lost with the resultant overgrowth of immunoresistant melanoma cells.[21]

Monoclonal antibodies

Antitumor monoclonal antibodies (mAb) are an alternative form of effector immunotherapy. Rituximab (anti-CD20 mAb) and Herceptin (anti HER-2 Neu mAb) are successfully used in the treatment of B-cell non-Hodkin's lymphoma (NHL) and breast carcinoma patients, respectively.[3] There are increasing numbers of novel antitumor mAb that are tested in preclinical and first-phase clinical trials.[33] Rituximab (MabThera®) is a chimeric IgG1 kappa mAb, produced by recombinant gene technology methods. It is used in the treatment of III-/IV-stage chemoresistant follicular NHL and its relapses.[34] CD20 antigen is expressed by healthy B-lymphocytes and more than 90% of B-cell NHLs, but not by plasma cells. Rituximab quickly depletes CD20+ B-lymphocytes with the restoration of their numbers only 9 to 12 months after treatment.[34] Mechanisms involved in the depletion of B lymphocytes are antibody mediated cell cytotoxicity, complement dependent cytolysis and induction of apoptosis.[34] As the plasma cells are not affected, the production of immunoglobulins is practically normal.[34] Monoclonal antibody efficiency can be improved by their conjugation with toxins, radionuclides or cytotoxic drugs. Mylotrag, anti-CD33 immunotoxin, is used in the treatment of CD33 positive acute myeloic leukemia in older patients and shows comparable antileukemic efficiency to chemotherapy with fewer side effects.[3]

Conclusion

There are three main requirements for cancer immunotherapy to be effective. *First*, there must be enough high-affinity tumor-specific lymphocytes; *second*, tumor-specific lymphocytes must successfully infiltrate the tumor and *third*, the tumor infiltrating lymphocytes must effectively kill tumor cells. Real situation is totally different. *First*, potentially tumor-reactive T-lymphocyte population is represented by a small number of low-affinity T-lymphocytes. Tumor vaccines can elicit only weak immune response against tumor antigens. *Second*, local tumor microenvironment is an important barrier for T-lymphocyte infiltration of the tumor. *Third*, tumor cells develop several strategies to evade successfully antitumor immune response. Although tumor vaccines arose as promising anticancer strategy with several potential advantages over standard anticancer regimens, the results of clinical trials on tumor vaccines have so far been disappointing. Considering all the barriers that the immune system must overcome to reject the tumor, disappointing results are all but surprising.

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Preoperative concomitant chemoradiotherapy in esophageal cancer

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Background. *Currently primary treatment options for esophageal cancer are surgery only or concomitant chemoradiotherapy (CRT) and the long-term survival of patients with locally advanced disease is rare. Preoperative concomitant CRT seems to be beneficial, mostly in patients who achieve a complete pathologic response (pCR) after CRT. In this retrospective analysis the efficiency and toxicity of preoperative CRT in patients with locally advanced esophageal cancer was analysed as well as the influence of pCR on the survival.*

Patients and methods *From 1996 to 2002 41 patients with locoregionally confined esophageal cancer were treated with cisplatin 75 mg/m^2 and 5-FU 1000 mg/m^2 as 4 day contonuous infusion starting on days 1. and 22. with concomitant radiotherapy 4500 cGy, 200-300 cGy/day. Esophagectomy followed 4-5 weeks after radiotherapy. After the surgery patients were followed-up regularly at 3-6 months intervals.*

Results. *The pCR was achieved in 26.8% of patients. The overall median survival time was 18 months for all patients, 21.2 months for patients who achieved pCR and 16 months in those with residual disease (p= 0,79). Postoperative mortality rate was 22%. The median dose intensity for cisplatin was 92% and for 5-FU 71.5% of the planned dose. Disease recurred most often locoregionally (31.7%) and the overall recurrence rate was 43.9%.*

Conclusion. *Modern radiation techniques and the adequate dose intensity could further improve the loco-regional control. The selection of patients without comorbid conditions and without already present distant metastases is essential for this combined treatment approach.*

Key words: esophageal neoplasms – drug therapy - radiotherapy

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Introduction

In the last few decades the incidence of esophageal cancer is constantly growing in Western Europe and USA where adenocarcinoma represents 60% of all esophageal carcinomas.[1,2] According to data of the Cancer registry of Slovenia the incidence

of esophageal cancer has risen in recent years (76 patients in 1998, 100 patients in 2002) but adenocarcinoma still represents only 14% of all histological confirmed cancers and most of them are squamous cell carcinomas.[3,4]

Primary treatment modalities include surgery alone or concomitant chemoradiotherapy. The surgical treatment is a standard treatment for stage I, II and III-T3 (www.cancernet.nci.nih.gov) and is feasible in 40-60% of patients.[5,6] In 75% of patients esophageal cancer is diagnosed when already locally advanced (stage IIB, III). The postoperative mortality rate is 10-15%, in experienced centers less than 5%.[7] Postoperatively locoregional recurrence occurs in 30-60%.[8-10] After the only primary surgical treatment, 5-year survival for stage I disease and locally advanced disease is 50-80% and 5-10%, respectively.[11-13] Concomitant chemoradiotherapy was superior when compared to radiotherapy alone in the primary treatment of locally advanced esophageal cancer.[14,15] Preoperative radiotherapy does not improve outcome in comparison to surgery alone.[16-21]

Combined modality therapies (preoperative chemotherapy, preoperative concomitant chemoradiotherapy) are still under the clinical evaluation. According to the results from randomized trials, the role of preoperative chemotherapy is still inconclusive.[9,22-24] In nonrandomized clinical trials with preoperative concomitant chemoradiotherapy (CRT) a pathologic complete response (pCR) was achieved in average in 32% (11%-76%) of patients and predicted a better survival. The survival of patients with pCR at 3-years and 5-years was 29-92% and 20-71%, respectively. The survival of patients who did not achieve pCR at 3-years was 23-33%. The disease recurred in 46% of all patients. In patients with a pCR the disease recurred in 20%, mostly (80%) as distant metastases.[25] In one randomized clinical trial the concomitant preoperative CRT showed some modest survival benefit over surgery alone[26] but there was no benefit in other randomized trials. [10,20,27-29] The inconsistency of these results might be due to heterogeneous patients' population, tumours characteristics and different treatment protocols.

In this retrospective study we analyzed the efficacy and toxicity of preoperative concomitant CRT in our patients with locally advanced esophageal cancer.

Patients and methods

Patients

Medical records of patients with esophageal cancer treated with preoperative concomitant CRT from 1996 to 2002 at the Institute of Oncology Ljubljana and Department of Thoracic Surgery Ljubljana were reviewed. Patients with histological confirmed locoregionally confined esophageal carcinoma (stage II and III), performance status < 2 according to WHO, adequate function of bone marrow, liver and kidney and absence of other malignances in their medical history with the exception of skin carcinoma were eligible. Staging of the tumour was based on the results of physical examination, blood tests, chest radiography, ultrasonography of abdomen, computed tomography of chest and upper abdomen, esophagogastroscopy with biopsy, liquid oral contrast examination of esophagus, endoscopic ultrasound of esophagus and bronchoscopy in patients with tumours in the middle and the upper third of esophagus.

Treatment

Patients were treated with concomitant preoperative chemotherapy (cisplatin 75 mg/m^2 days 1, 22 and 5-FU 1000 mg/m^2 days 1 - 4 and 22 – 25) and concomitant ra-

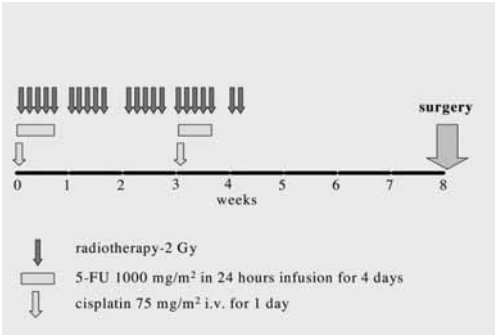

Figure1. Treatment plan with preoperative concomitant chemoradiotherapy.

diotherapy (4500 cGy, 200 – 300 cGy /day, field designs were either two or three-field plans on the linear accelerator, the radiation field included primary tumour with 5-cm superior and inferior margins and 2 cm lateral margins) (Figure 1). Esophagectomy followed 4 to 5 weeks after radiotherapy. After the surgery patients were followed–up regularly at 3-6 months intervals.

Statistical analysis

A statistical analysis with descriptive statistics and survival times and curves was performed using SPSS 10.0 for Windows statistical software. The survival was calculated according to Kaplan-Meier method and compared by a log-rank test between groups. The overall survival was estimated from the date of diagnostic biopsy to death and the relapse-free survival from the date of diagnostic biopsy to the first event of recurrence (local, regional, distant or their combinations).

Results

Forty-one patients (38 men, 3 women) with locoregionally confined esophageal cancer and without other comorbid conditions were treated with preoperative concomitant CRT in the period 1996 to 2002. The median age was 60 years (range 41-74). Squamous cell carcinoma was present in 39 (95.2%) patients and adenocarcinoma and poorly differentiated carcinoma in 2 (4.8%) patients. Tumour was located in the upper third of esophagus in 2 (4.9%) patients, in the middle third in 22 (53.6%) patients and in the lower third in 17 (41.5%) patients (Table 1).

Table 1. Patients and tumors characteristics

CHARACTERISTIC	No. of patients
TOTAL	41
MALES	38 (92,5%)
FEMALES	3 (7,5%)
MEDIAN AGE (min - max)	60 (41-74)
HISTOLOGY	
squamous cell	39 (95,2%)
adenocarcinoma	1 (2,4%)
poorly differentiated carcinoma	1 (2,4%)
TUMOR LOCALIZATION	
upper third	2 (4,9%)
middle third	22 (53,6%)
lower third	17 (41,5%)

Median dose intensity of received cisplatin was 23,2 mg/m^2/week (range, 16-34.2 mg/m^2/week) and of 5-FU 954.1 mg/m^2/week (range, 231.5-1394 mg/m^2/week) comprising 92.8% and 71.5% of the planned dose intensity, respectively. Patients who received in average less than 80% of planned dose intensity of both cisplatin and 5-FU had higher recurrence rate than patients who received 80% or more of the planned dose intensity (50% *vs* 30.8%; difference not statistically significant). Both, distant and locoregional recurrences were more common in patients with lower dose intensity, 28.6% vs. 7.7% and 35.7% vs. 23.1%, respectively (Table2).

Transthoracic esophagectomy (Lewis) and transhiatal esophagectomy were performed in 38 patients and 3 patients, respectively. R0 resection (microscopically free margins) was achieved in 39 patients and R1 resection (microscopically residual

Table 2. Chemotherapy (ChT) dose intensity and recurrence rates

RECURRENCE	No. of patients (%) DOSE INTENSITY OF ChT	
	< 80%	≥80%
LOCOREGIONAL	6/28 (21,4%)	3/13 (23,1%)
DISTANT	4/28 (14,3%)	1/13 (7,7%)
LOCOREGIONAL+DISTANT	4/28 (14,3%)	0
TOTAL	14/28 (50%)	4/13 (30,8%)

disease) in 2 patients. Postoperatively 9 patients (22%) died in 30 days. All patients died in a septic shock with multiorgan failure. Another 10 patient had nonfatal postoperative complications: pneumonia, empyema, necrosis of the stomach wall (fundus), fistula of the anastomosis and hylothorax. In patients with carcinoma located in the upper and middle third of the esophagus both fatal and nonfatal postoperative complications were more common than in patients with their carcinoma in the lower third, 25% vs. 17.6% and 29.2 vs. 17.6%, respectively (Table 3). In 4 out of 5 patients with necrosis of the stomach wall and in 3 out of 4 patients with fistula of anastomosis the tumour was present in the middle third of the esophagus.

A pathologic complete response (pCR) was achieved in 11/41 (26.8%) patients, in the upper two thirds of esophagus in 7/24 patients (29.1%) and in the lower third in 4/17 (23.5%) patients. Postoperatively 2 patients with pCR died. After a thorough lymph nodes examination by the pathologist metastatic disease was found in 9 patients (3 patients M_{1a}, 6 patients M_{1b} disease) and during the follow-up only 5 of these patients relapsed.

The median follow up was 40 months (6-52 months). The overall risk for recurrence was 43.9% (9 recurred locoregionally, 5 distant and 4 locoregionally and distant). Patients with and without pCR had a similar risk for recurrence (45.5% vs. 43.3%).

The median time to relapse was 21.5 months (95% CI: 7.3 – 35.7 months) and the median overall survival time was 18 months (95% CI: 10.8 – 25.1 months). Overall 2-year and 3-year survival was 36% and 28% respectively (Figures 2, 3). Patients with pCR had the median survival time of 21.2, months (95% CI: 2.4-40 months) and patients without a pCR 16 months (95% CI: 7.6-24.4 months, p=0.79).

Discussion

In this retrospective analysis of our patients with locally advanced esophageal cancer treated with preoperative concomitant CRT the pathologic complete response rate (26.8%), recurrence rate (43.9%) and

Table 3. Tumour localization and postoperative complications

POSTOPERATIVE COMPLICATIONS	No. of patients (%) TUMOR LOCALISATION	
	UPPER+MIDDLE THIRD	LOWER THIRD
NONE	11/24 (45,8%)	11/17 (64,7%)
NON-FATAL	7 /24(29,2%)	3/17 (17,6%)
FATAL	6/24 (25%)	3/17 (17,7%)
TOTAL	24	17

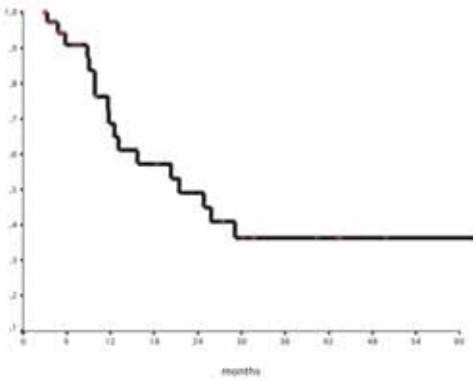

Figure 2. Relapse-free survival (n=41).

median overall survival time (18 months) are comparable with the results of published trials.[25]

In the majority of published clinical trials, the survival of patients who achieved a pCR was significantly better than of those without a pCR. In our study the median survival of patients with a pCR (21.2 months) was also better than in those without a pCR (16 months) but the difference was not statistically significant. The main reason could due to small number of patients included in our study.

We also observe a high postoperative mortality rate and efforts to reduced postoperative mortality could further improve the overall survival. Necrosis of stomach wall occurred in 5 patients and led to death in 2 patients. This treatment complication is not listed among the common in the literature. The postoperative mortality rate was higher in patients with tumours located in the upper two thirds of esophagus in comparison to the lower third (27.3% vs. 17.6%) but the difference was not statistically significant. Altogether, 80% of all stomach wall necrosis and 75% of all fistulas on the anastomoses occurred in patients with tumours in the upper two thirds. Therefore, concomitant CRT without surgery might be a reasonable option for patients with cancer localized in the upper two thirds of the esophagus. Although there are no randomized studies comparing surgery versus concomitant CRT alone, the survival of patients in the concomitant CRT arms (in some randomized clinical trials comparing concomitant CRT versus radiotherapy) is similar to the survival of patients treated in the trials comparing surgery and preoperative concomitant CRT plus surgery. This comparison is speculative and not evidence based, but it might be reasonable to adopt it for the subgroup of patients with high mortality rate after the surgery as are in our case the patients with their cancers in the upper two thirds. Improved surgical techniques and more intense postoperative care are also important options for these patients since the surgery was beneficial in 5 out of 9 our patients with residual carcinoma in the lymphnodes (M_{1a} and M_{1b} disease) after concomitant CRT who are still free of recurrence after the median time of follow up of 40 months. Currently it is also hard to predict who is going to achieve a pCR after concurrent chemoradiotherapy and the achieved pCR rates are relatively low. For these reasons the role of surgery remains an important part of this multimodality treatment approach.

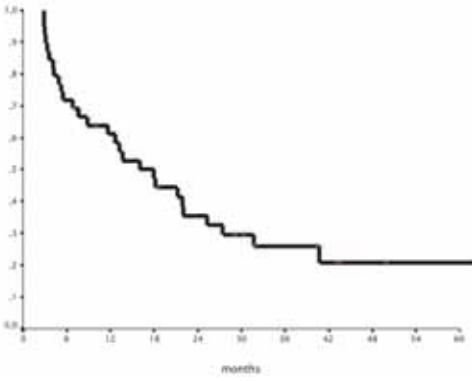

Figure 3. Overall survival (n=41).

The median dose intensity for cisplatin and 5-FU was 92.8% and 71.5% of the

planned dose intensity, respectively. The main reason for the lower dose intensity for 5-FU might be due to well known higher incidence of mucositis in case of concurrent chemoirradiaton. Patients with median dose intensity of less than 80% for both cisplatin and 5-FU had a higher locoregional recurrence rate (35.7% *vs* 21.4%) and increased incidence of distant failure by almost four-fold (28.6% *vs* 7.7,%). Therefore it seems important that the dose intensity is delivered as planned in the schedule. It seems that this is feasible, since in our study the postoperative complication rates (including fatal) were similar regardless of the dose intensity received. Modern three dimensional conformal radiotherapy planning could be of additional benefit for the locoregional control.

An extremely important issue is the selection of patients for this combined modality treatment. We should exclude patients in poor performance status with distant metastases who will not benefit with this kind of treatment.

Conclusion

Preoperative concomitant CRT might be beneficial at least in a subset of patients with locally advanced esophageal cancer in good performance status and without important comorbidity. For tumours originating in the upper two thirds of esophagus the role of surgery should be used in highly selected cases. A multidisciplinary approach of surgeons, radiation oncologists and medical oncologist is essential.

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Body mass index and lung cancer risk in never smokers

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Background. *A relationship between body mass index (BMI) and lung cancer risk in never smokers has not been reported precisely. To evaluate the risk of lung cancer associated with BMI in never smokers, we conducted a case-control study.*

Methods. *The relationship between BMI and the risk of lung cancer in never smokers was investigated in a study of 204 lung cancer cases and 398 controls admitted between 1987 and 2005. Controls were selected from hospitalized age-matched never-smoking patients with non-malignant respiratory disease.*

Results. *When compared with BMI of the leanest group (BMI<20.8) in men, no inverse association between BMI and lung cancer was observed after the adjustment for age (the second BMI group: BMI≥ 20.8 to < 22.9; p=0.683, the third BMI group: BMI≥ 22.9 to < 24.9; p=0.745, and the highest BMI group: BMI≥ 25.0; p=0.327). Similarly, no association in women was found between BMI and lung cancer in these three BMI groups (the second group, p=0.639; the third group, p=0.667; the highest group, p=0.978) when compared with that of the leanest BMI group.*

Conclusions. *Our present study indicated that the association between leanness and the risk of lung cancer might be influenced by other factors such as smoking.*

Key words: lung neoplasms – epidemiology; risk factors; adenocarcinoma; body mass index; smoking

Introduction

An elevated risk of lung cancer associated with lower levels of body mass index (BMI) has been reported in previous studies.[1-9] However, the interpretation of the association between low BMI and lung cancer is complicated by the fact that weight loss may be a sign of smoking. In general, smokers tend to be lighter than non-smokers,[1, 10-15] and it is believed that nicotine is responsible for the effect of smoking on body weight because nicotine appears to increase the metabolic rate.

We have recently performed a case-control study of subjects participating in a mass-screening program and found an increased risk of lung cancer for lower BMI in male patients.[16] Most of them were current smokers.[16] However, the inverse

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association between the risk of lung cancer and BMI was not found in female patients. This was inconsistent with the results of previous studies.[1-9] The difference between the findings of previous studies and our own was most probably due to difference in study population. The subjects in previous studies were symptomatic, but all the subjects in our recent study were asymptomatic preclinical patients.[16] In addition, there were higher proportions of never-smoking women and lung adenocarcinoma in our study, and the different results might also be influenced by them.

Whereas, it remains an unsettled question whether there is an inverse association between the risk of lung cancer and BMI among never smokers, especially in those with lung adenocarcinoma. In order to evaluate the association between BMI and the risk of lung cancer in patients who never smoked, we conducted a hospital-based case-control study. In this study, we also estimated the association between BMI and the risk of lung adenocarcinoma in never-smokers.

Methods

Study Design

A hospital-based case-control study was conducted from January 1987 to September 2005 in Respiratory Division of Tsukuba University Hospital, to explore the risk of lung cancer associated with lower levels of BMI at the time of initial diagnosis.

Patients

All cases and controls were recruited at Tsukuba University Hospital and were identified from the medical record. All patients who were histopatologically confirmed to have lung cancer were included in this analysis. Pathological diagnoses were based on the 1999 WHO classification of lung neoplasms. Only lung cancer patients as well as control subjects, who had never smoked and without occupational or domestic exposure to other recognized carcinogens, were included in this study. The hospital-based controls matched by age (±5 yr), gender, and time of hospitalization were recruited from our division, including inpatients with non-malignant respiratory diseases. Patients with other cancer at any site were excluded. This study was approved by the institutional ethics committee of University of Tsukuba.

Statistical analysis

BMI was calculated from body height and weight, which were measured and reported by nurses at the time of admission, using the formula for Quetelet's index (expressed in kg/m^2). BMI was categorized into four levels on the basis of the distribution in the total study population ($BMI < 20.8$, leanest; $20.8 \leq BMI < 22.9$, second; $22.9 \leq BMI < 25.0$, third; $25.0 \leq BMI$, highest), which was the same category as our previous study based on the results of community mass screening.[16] We used the leanest category ($BMI < 20.8$) as the reference group for analyses.

Logistic regression was used to examine the effect of BMI on lung cancer risk. Results with a p value less than 0.05 were regarded as significant. The software package SSPE (SSPE Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA) were used to perform the analyses stated above.

Results

During the study period, 919 patients with primary lung cancer were diagnosed in our division. Among them, a total of 204

(22.2 %) documented never-smokers with primary lung cancer were registered.

Table 1 shows the characteristics of 204 lung cancer cases. Three-fourths of all cases were women. Although the age range for this study was between 22 and 85 years, median age of the male and female patients was 64 and 67 years, respectively. Twenty-four (64.9 %) of 37 male patients, and 151 (90.4 %) of 167 female patients had lung adenocarcinoma. There was no patient with small cell lung cancer in both genders (Table 1). Among the 204 patients, 81 had stage IA-IIIA, 35 had stage IIIB, and 88 had stage IV disease.

Table 1. Characteristics of 204 lung cancer patients

Characteristics	Men	Women
Number of patients	37	167
Age (median, range), Yr	64, 22 – 80	67, 35 – 85
Histologic types		
Adenocarcinoma	24	151
Squamous cell carcinoma	10	9
Large cell carcinoma	1	4
Others	2	3
Stage		
IA – IIIA	11	70
IIIB	11	24
IV	15	73

Table 2 shows odd ratios and 95 percent confidence intervals for the association between the risk of lung cancer and lower levels of BMI at the time of diagnosis with lung cancer. When compared with BMI of the leanest group (BMI<20.8) in men, no inverse association between BMI and lung cancer was observed after the adjustment for age (the second BMI group: BMI≥ 20.8 to < 22.9; p = 0.683, the third BMI group: BMI≥ 22.9 to < 24.9; p = 0.745, and the highest BMI group: BMI≥ 25.0; p = 0.327). In women, no association was found between BMI and lung cancer in these three BMI group (the second BMI group, p = 0.639; the third BMI group, p = 0.667; the highest BMI group, p = 0.978) when compared with that of the leanest BMI group. In male adenocarcinoma, the second BMI group (p=0.967), the third BMI group (p=0.310), and the highest BMI group (p = 0.378) did not exhibit higher odd ratio than that of the leanest BMI group after the adjustment for age.

As above mentioned, 175 of 204 patients were adenocarcinomas. There were too-few

Table 2. Association of BMI with lung cancer

BMI	Case Patients, No.	Control Subjects No.	OR	95% CI	p-value
Men					
< 20.8	13	32	1.0		
20.8 – 22.8	10	20	0.8	0.3 – 2.2	0.683
22.9 – 24.9	5	10	0.8	0.2 – 2.8	0.745
≥ 25.0	9	13	0.6	0.2 – 1.7	0.327
Women					
< 20.8	67	131	1.0		
20.8 – 22.8	43	75	0.9	0.6 – 1.4	0.639
22.9 – 24.9	23	51	1.1	0.6 – 2.0	0.667
≥ 25.0	34	66	1.0	0.6 – 1.7	0.978

OR: odd ratio; CI: confidence interval

Table 3. Association of BMI with lung adenocarcinoma

BMI	Case Patients, No.	Control Subjects No.	OR	95%CI	p-value
Men					
< 20.8	8	26	1.0		
20.8 – 22.8	6	19	1.0	0.3 – 3.5	0.967
22.9 – 24.9	4	6	2.2	0.5 – 9.6	0.310
≥ 25.0	6	11	1.7	0.5 – 6.3	0.378
Women					
< 20.8	61	120	1.0		
20.8 – 22.8	36	67	1.1	0.6 – 1.8	0.831
22.9 – 24.9	22	46	0.9	0.5 – 1.7	0.841
≥ 25.0	32	58	1.1	0.6 – 1.8	0.762

OR: odd ratio; CI: confidence interval

patients to stratify the histologic subtypes in both men and women, therefore, we examined the association only in adenocacinoma cases (Table 3). In men, the second BMI group (p = 0.967), the third BMI group (p = 0.310), and the highest BMI group (p = 0.378) did not exhibit higher odd ratio than that of the leanest BMI group after the adjustment for age. In women, no association was found between BMI and lung cancer in these three BMI groups (the second BMI group, p = 0.831; the third BMI group, p = 0.841; the highest BMI group, p = 0.762) when compared with that of leanest BMI group. For adenocarcinoma, therefore, the inverse association was not observed in both genders.

Discussion

An inverse gradient between BMI and the incidence of lung cancer has been reported in several case control and cohort studies.[1-9] However, the interpretation of the association between low BMI and lung cancer is complicated by the fact that low BMI may be influenced by other factors such as smoking. It is possible that smokers tend to have lighter body weight, possibly a consequence of the metabolic effects of nicotine.

In order to investigate the association between lower levels of BMI and the risk of lung cancer in never-smoking patients, we, therefore, conducted a hospital-based case-control study.

The results of the present study indicate two important points. The first point of importance is that we found the absence of an inverse gradient between BMI and the risk of lung cancer in never-smoking female patients, which was consistent with our recent study of subjects participating in a mass screening program.[16] Interestingly, Rauscher *et al* reported an elevated risk of lung cancer associated with not "low" but "high" levels of BMI in non-smoking female patients.[17] The difference between the findings of Rauscher and our own was probably due to different study populations. All patients and controls in our study were never-smokers. On the other hand, however, not all subjects in the study by Rauscher *et al* were never-smokers.[17] They included 188 patients who haven't smoked more

than 100 cigarettes in their lifetime and 224 patients who haven't smoked more than 100 cigarettes during the last 10 years.[17] Therefore, there was a possibility that the results were influenced by the residual effects of smoking.

The majority of published results investigating the association between lower BMI and lung cancer risk were based on studies conducted in Western countries where the prevalence of obesity is high.[2,8,17] The difference in categorized BMI levels might also influence the difference between the findings of previous authors and our own. The second important point is that both thin male and female never smokers did not have an increased risk of lung adenocarcinoma. The results of the present study indicate that lower BMI is not significantly associated with the risk of lung adenocarcinoma. Some lung adenocarcinomas in never smokers can arise without the growth promoting effects of the carcinogens present in cigarette smoke,[18] one can postulate that the mechanism of carcinogenesis of adenocarcinomas arising in smokers and never-smokers may be different, and these distinct tumorigenic mechanisms can imply differences in tumor biology, demographic characteristics as Brownson and colleague suggested.[19]

Although we showed the above-mentioned two findings of importance, we must acknowledge the limitation of this study. First, it was a hospital-based case-control study. As with any hospital-based case-control study, it has been suggested that such case-control studies may reflect the presence of disease other than lung cancer in control subjects.[2] The second limitation of the present study is that it included only small number of patients and controls in a single institute. The third, 123 (60.3%) of 204 patients had locally advanced or metastatic lung cancer and stage of the disease would be important in that more advanced disease might itself be associated with weight loss. It is interesting to know the association between BMI and the risk of lung cancer among patients with early disease, but we could not evaluate the association because of small number of study population. The last, we could not examine the impact of environmental tobacco smoke (ETS) exposure on this association. An accurate assessment of the amount of ETS exposure in never smokers is necessary for determining the lung cancer risks associated with ETS exposure.

It is well known that smokers tend to be leaner than non-smokers.[1,10-15] Several previous studies have reported an association between leanness and risk of lung cancer, mainly among smokers,[12,14] and among men with smoking-related disease.[14] Inconsistence with previous studies,[1-9] our results indicated the possibility that the previously reported association between leanness and the risk of lung cancer might be influenced by other factors such as smoking. A large cohort study will be needed to confirm the current results.

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Schedule-dependency of doxorubicin and vinblastine in EAT tumours in mice

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Background. *Antitumour schedule-dependency of the doxorubicin and vinblastine combination was explored.*

Materials and methods. *Intraperitoneal Ehrlich ascites tumours (EAT) syngeneic to CBA mice were treated with vinblastine or doxorubicin alone, or in combined treatment schedules.*

Results. *Combinations of doxorubicin and vinblastine administered at 48-h, but not at 24-h interval, regardless of the sequence of drugs, significantly reduced the number of tumour cells in the ascites in comparison with all other treatments. In the combined treatment schedules, the predominant morphological changes as well as DNA distribution pattern were dependent on the first drug applied. Regardless of the sequence of the drugs, median survival times of animals did not significantly differ between the treatment groups.*

Conclusions. *The effect of combination of vinblastine and doxorubicin is schedule-dependent. The time interval, but not the sequence of drugs seems to be crucial for the observed effect. The data from preclinical studies are important for planning combined treatment schedules in clinical setting.*

Key words: carcinoma Ehrlich tumor – drug therapy; doxorubicin; vinblastine; drug administration schedule

Introduction

Doxorubicin (Doxo) and vinblastine (VLB) or their analogues are used in combined treatment schedules for a variety of malignant tumours, *i.e.* breast, ovarian, lung, urothelial cancer and Hodgkin disease.[1-6] The combination chemotherapy is used with the aim to enhance antitumour efficacy. In planning chemotherapy protocols in patients, drugs with different mechanisms of action, non–cross-resistant and with non-overlapping toxicities are used. In planning the intervals between sequential cycles of chemotherapy mostly the tolerance of bone marrow is taken into consideration. However, in clinical setting, attention is rarely paid to possible drug interference and schedule dependency of the drug combinations. There are usually no data on the sequence and exact timing of particular drugs in combined chemotherapy

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protocols. Schedule dependency and drug interference has been extensively studied in tumour models.[7-11] The implementation of results from preclinical studies could make planning of treatment schedules more rational and thus improve the effect of chemotherapy in patients.

In our clinical research on the combined treatment of anaplastic thyroid carcinoma, VLB or Doxo combined with radiation was a promising treatment for achieving local control of the primary tumour.[12] A logic further development of treatment of this very aggressive tumour would be a schedule combining both Doxo and VLB with radiation. In our previous preclinical and clinical studies, we demonstrated that, after pretreatment of tumour cells with VLB, the accumulation of bleomycin or cisplatin in the tumour cells was increased.[13,14] The increased cell membrane permeability and consequently a better penetration of the drug into the cell was the proposed mechanism of action. It would be clinically relevant to explore if pretreatment with VLB also influences the accumulation of Doxo in the tumour cells.

The primary objective of the current work was to explore in a preclinical study whether there is a schedule-dependency of the combination of VLB and Doxo. The second objective was to find out whether, after pretreatment with VLB, the accumulation of Doxo within the tumour cells could be increased.

Materials and methods

Drug formulation

VLB (Vinblastine sulphate, Lilly France S.A., Fagersheim, France) was dissolved in 0.9% NaCl solution at a concentration 2.5 μg/ml. Doxorubicin (Doxo; Doxorubin, Pharmachemie B.V., Haarlem, The Netherlands) stock solution (2 mg/ml) was further diluted in 0.9% NaCl solution to achieve doses of 0.9 to 3.6 mg/kg. Each animal was injected i.p. with adjusted volume (approx 0.5 ml) of drug solution to achieve VLB dose of 62.5 μg/kg and different Doxo doses ranging from 0.9 to 3.6 mg/kg. This low VLB dose was selected according to our previous studies where we demonstrated that this dose significantly affected cell membrane fluidity with a minimal effect on cell survival.[15,16] All doses used were far below the maximal tolerated dose level.

Animals

Inbred CBA mice were purchased from the Institute of Pathology, Medical Faculty Ljubljana (Slovenia). Mice were maintained at a constant room temperature (22°C) and natural day/night light cycle in a conventional animal colony. Before experiments, mice were subjected to an adaptation period of at least 10 days. Mice of both sexes, in good condition, weighing 22-30 g, without signs of infection, 10-15 weeks old, were included in the experiments. Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia, permission No. 323-02-200/2004 and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Bethesda MD).

Tumour model

Intraperitoneal (i.p.) Ehrlich ascites carcinoma (EAT) syngeneic to CBA mice was used in the study. The tumour was maintained i.p. as ascites by serial transplantations once a week. For the transplantation of i.p. tumours, the tumour cells from the donor mouse were harvested by peritoneal lavage with 4 ml of 0.9% NaCl solution, washed and resuspended at a concentration of 3×10^6 cells/ml. The tumours were transplanted by i.p. injection of 1.5×10^6

viable EAT cells in 0.5 ml 0.9% NaCl solution. Cell viability, determined by Trypan dye exclusion test, was over 95%.

Treatment protocol

In the first part of the study, different doses of Doxo (0.9, 1.8 and 3.6 mg/kg) were tested in order to determine the cell survival, accumulation of Doxo in the tumour cells and effects of Doxo on DNA distribution. Three days after tumour transplantation, the animals were treated with Doxo and thereafter sacrificed at different post treatment intervals (24, 48 and 72 h) to evaluate the treatment effectiveness.

In the combined treatment schedule, the animals were randomly allocated three days after tumour transplantation into the following groups: control (i.p. treated with 0.9% NaCl solution), VLB alone, Doxo alone, VLB followed by Doxo, Doxo followed by VLB and both drugs given simultaneously. The time interval between i.p. injection of the first and second drug was 24 or 48 h. When the chemotherapy with VLB or Doxo was tested alone, 0.9% NaCl was injected 24 h or 48 h afterwards, as a sham intervention (Table 1). The mice were sacrificed 24 h after the completion of therapy by cervical dislocation. Each experimental group consisted of at least 3 mice and the data were pooled from 2-3 independent experiments.

Cell number, flow cytometric analysis of DNA content, Doxo fluorescence and cell morphology

Twenty-four hours after the completion of therapy, the mice were sacrificed and tumour cells were harvested by peritoneal lavage with phosphate-buffered saline supplemented with 20% bovine serum albumin. The tumour cells harvested from individual animals were used for the measurement of cell number, flow cytometric DNA measurements and cell cycle analysis, Doxo accumulation and study of cell morphology. The effect of different treatments on cell survival

Table 1. Treatment protocol. A – 24 h interval between treatments; B. 48 h –interval between treatments.

A

Group	Day 0	Day 3	Day 4	Day 5
VLB 24 h	Tum. inoculation	VLB	Physiological saline	Harvesting
Doxo 24 h		Doxo	Physiological saline	Harvesting
VLB + Doxo 24 h		VLB + Doxo		Harvesting
VLB 24 h Doxo		VLB	Doxo	Harvesting
Doxo 24 h VLB		Doxo	VLB	Harvesting

B

Group	Day 0	Day 3	Day 5	Day 6
VLB 48 h	Tum. inoculation	VLB	Physiological saline	Harvesting
Doxo 48 h		Doxo	Physiological saline	Harvesting
VLB + Doxo 48 h		VLB + Doxo		Harvesting
VLB 48 h Doxo		VLB	Doxo	Harvesting
Doxo 48 h VLB		Doxo	VLB	Harvesting

was determined by counting the tumour cells in the peritoneal lavage of the animals by means of haemocytometer. The results of cell number were presented as the percent of cells compared to the number of cells in the control, saline treated animals. For the flow cytometric DNA measurements, the cells were prepared according to a modified Otto method.[17] In brief, the cells were treated for 20 min with a solution consisting of 0.2 M citric acid and 0.5% Tween 20, and then fixed with 70% ethanol for at least 24 h. After the treatment with 0.5% pepsin (Serva, Heilderberg, Germany) for 5 min, the cells were stained with 4'6-diamidino-2 phenylindole (DAPI, Serva) for DNA. The measurements of DNA content of cells were performed using a PAS III (Partec, Münster, Germany) flow cytometer. The results were presented in the histograms of cell number against fluorescence. The data were analyzed with Multicycle AV (Phoenix Floe Systems, San Diego, CA) program. For flow cytometric measurement of Doxo accumulation in the tumour cells, the cells were centrifuged and resuspended in phosphate buffered saline at a concentration of $5x10^5$ cells/ml. The samples were analysed on FacsCalibur flow cytometer (BD PharMingen, San Jose, California, USA) using 585/42 bandpass filter and the results were presented in the histograms of cell number against fluorescence. In addition, the cell samples counterstained with DAPI to distinguish between dead cells (DAPI positive) and the cells with Doxo accumulation were studied on fluorescence microscope. Cell morphology was studied on the cell smears stained with Giemsa (Merck, Darmstadt, Germany).

Statistical analysis

The data are presented as arithmetic means ± SE (standard error of the mean). The significance of the effect was determined using post-hoc Tukey's t-test after One-way analysis of variance was performed; the levels of less than 0.05 were taken as indicative of significant differences. Survival curves were plotted by the Kaplan-Meier method. The differences between the survival curves were determined using Dunn's method after Kruskal-Wallis One way analysis of variance on ranks was performed. Statistical analysis was carried out using SigmaStat statistical software (SPSS, Chicago, USA).

Results

Effect of different Doxo doses in EAT tumour cells

The growth curves of the cells in the ascites of CBA mice treated with different Doxo doses did not differ up to 48 h post treatment. Only at 72 h post treatment, already the lowest dose of Doxo (0.9 mg/kg) induced a significant reduction of the number of cells in the ascites (Figure 1). The increase in Doxo dose resulted in an increased cytotoxicity at this time interval. However, there was no statistical difference between the two higher doses tested (1.8 and 3.6 mg/kg). Based on these results, the lowest, relatively non-cytotoxic Doxo dose (0.9 mg/kg) was chosen for the subsequent experiments combining VLB and Doxo. In order to detect a possible potentiation of Doxo or VLB cytotoxicity in combined therapy schedules, an excessive cell kill caused by higher dose of Doxo alone would not be desirable.

Beside cytotoxicity of different doses of Doxo, intracellular accumulation of Doxo, morphological changes and DNA distribution in EAT tumour cells were also studied. From the flow cytometric measurements of number of cells with internalized Doxo it was evident that the number of fluorescent cells did not differ between the doses tested at 24 and 48 h post treatment, whereas at 72 h post treatment, significantly less fluorescent cells were observed at two

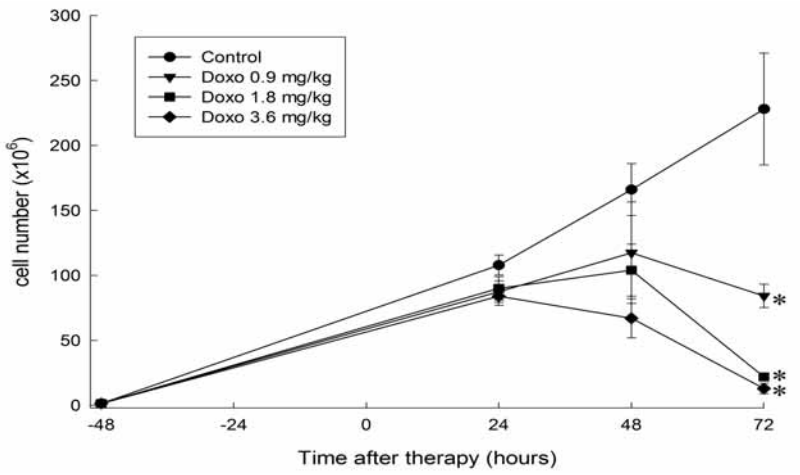

Figure 1. Cytotoxic effect of Doxo to EAT tumour cells in ascites. EAT tumour cell number as a function of time in the ascites of mice treated with different doses of Doxo injected i.p. Points are mean of 9 mice per group. *p<0.05 compared to control group.

higher doses because of significant cell kill induced by these doses (Figure 2).

However, the amount of Doxo in the tumour cells increased in a dose-dependent manner, as evident from the median value of the peak of fluorescence intensity of cells and fluorescence microscopy (Figure 3). Median value of the peak of fluorescence intensity at the lowest dose was 167 and increased to 320 at 3.6 mg/kg Doxo 24 h post treatment (data not shown).The cell cycle phase distribution in EAT tumour cells 48 h post treatment demonstrated that Doxo greatly reduced the number of cells in G_1 phase of cell cycle and caused a block in G_2M compartment (see Figure 6). The

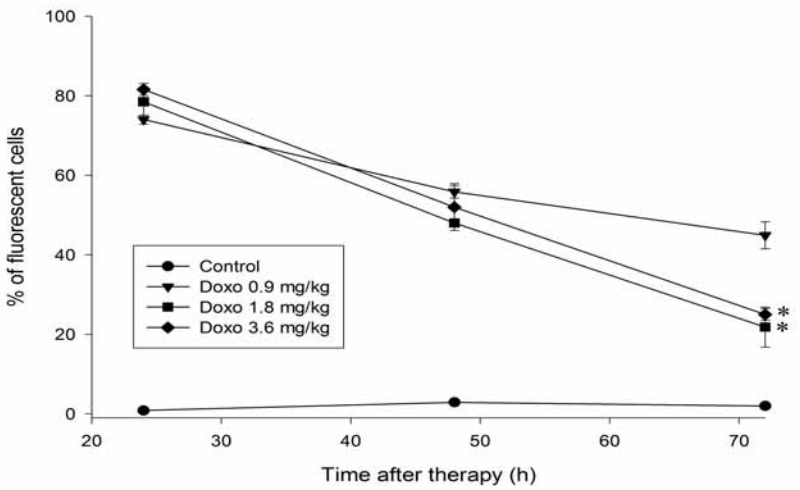

Figure 2. Percentage of EAT tumour cells with Doxo accumulation as determined from fluorescent histograms obtained by flow cytometer. Points are mean of 9 mice per group .* p<0.05 compared to treatment with Doxo dose of 0.9 mg/kg.

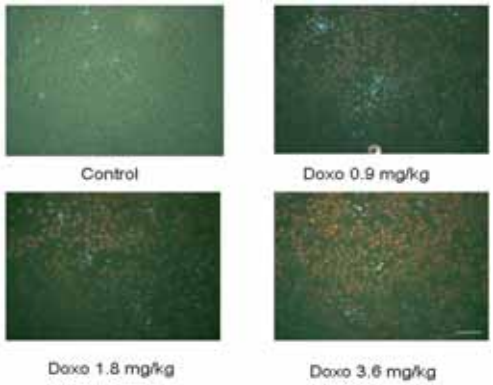

Figure 3. Fluorescence intensity in EAT tumour cells 24 h after treatment demonstrated increased amount of Doxo within the cells with increasing Doxo doses. Doxo positive cells – red, DAPI positive (dead) cells –blue (bar 100 μm).

morphological changes observed in the cell smears prepared from the same samples corresponded to the changes in cell cycle phase distribution. Enlarged cells with enlarged nuclei and nucleoli were observed at all doses tested compared to the untreated control cells. In addition, mitoses were rare and degenerative changes, such as poto-

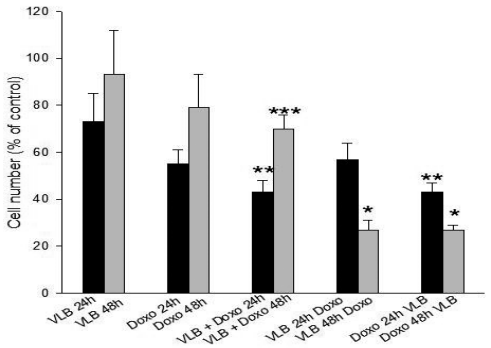

Figure 4. Survival of EAT tumour cells after treatment with 24-h (black bars) or 48-h (grey bars) interval between the treatments with Doxo and VLB. Pertinent control groups, *i.e.* treatment with either of the drugs alone and VLB + Doxo given simultaneously, are included. For protocol see Table 1. Cells from ascites were harvested 24 h after completion of treatment. Bars are mean of at least 6 mice per group. * p<0.05 compared to all treatment groups; ** p<0.05 compared to treatment with VLB, but not to treatment with Doxo; *** p<0.05 compared to control.

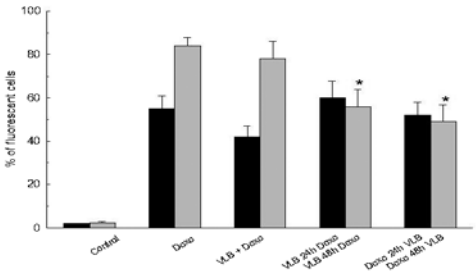

Figure 5. Accumulation of Doxo in EAT tumour cells. Percentage of Doxo positive EAT tumour cells after treatment with 24-h (black bars) or 48-h (grey bars) time interval between Doxo and VLB administration. Pertinent control groups, *i.e.* treatment with either of the drugs alone and VLB+Doxo given simultaneously, are included. For protocol see Table 1. Cells from ascites were harvested 24 h after completion of therapy. Bars are mean of at least 6 mice per group.* p<0.05 compared to treatment with Doxo and VLB + Doxo.

cytosis and fragments of cytoplasm, were observed at all doses tested (see Figure 7).

Effects of different VLB and Doxo treatment combinations delivered with a 24-h interval between the administration of the two drugs in EAT tumour cells

To determine the effect of VLB and Doxo combinations delivered with a 24-h interval between the two drug administrations on the survival of EAT tumour cells, the cell number was determined in the ascites. All the treatments significantly reduced the EAT tumour cell number in the ascites compared to the untreated control animals. However, there was no significant reduction in cell number following the treatment with different treatment combinations compared to the treatment with Doxo alone. The treatment combinations in either of the schedules reduced the survival only to the level of the survival induced by Doxo alone (Figure 4). In contrast, compared to the treatment with VLB alone, the treatment combinations with two drugs, injected simultaneously or with Doxo preceeding

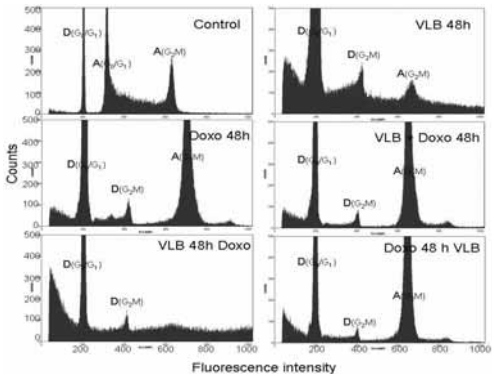

Figure 6. The DNA distribution in EAT tumour cells after treatment with different VLB+Doxo treatment schedules, with the 48-h interval between the injections of drugs. D-diploid value of DNA (inflammatory cells); A – aneuploid value of DNA (tumour cells).

VLB resulted in significantly reduced cell number (Figure 4).

The percentages of fluorescent (Doxo positive) cells measured 24 h after the completion of treatment were the same in all groups regardless of the treatment schedule (Figure 5).

The DNA distribution measurement of tumour cells from the animals treated with Doxo alone showed an increased G_2M compartment of the cell cycle. In the cytological smears (Doxo 24 h) prepared from the same samples as for the DNA measurements, enlarged cells with enlarged nuclei and nucleoli were observed. Mitoses were very rare. The same effects on the cells were seen also in the samples when Doxo preceded VLB for 24 h as well as when the drugs were given simultaneously. Rare mitoses in cytological smears together with enlarged G_2M compartment in DNA histograms speaks for a block in G_2 phase of the cell cycle.

The DNA distribution measurements of the cells treated with VLB alone (VLB 24 h) or when VLB preceded Doxo for 24 h showed the cells with very high DNA values, but with no distinctive peaks (data not shown). The cells taken from the same samples were enlarged, but to the lesser degree than the

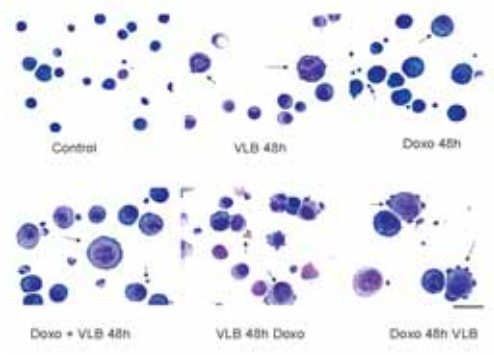

Figure 7. Morphological changes in EAT tumour cells after treatment with different VLB+Doxo treatment schedules, with the 48-h interval between the injections of drugs. (Giemsa staining, bar 50 μm). Arrows indicate enlarged cells, potocytosis and fragments.

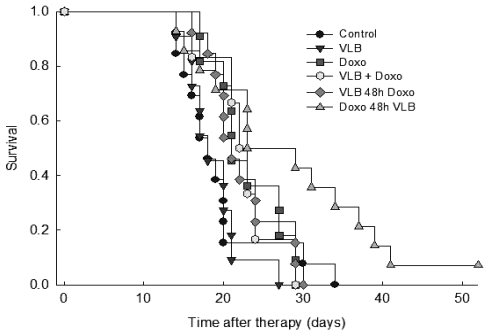

Figure 8. Animal survival after treatment with different VLB+Doxo treatment schedules, with the 48-h interval between the drugs injections.

cells treated with Doxo. Multinucleated cells and cells with irregular mitoses were also observed (data not shown).

In cytological smears of all treatment groups, a high percentage of inflammatory cells was observed, which was also shown in DNA histograms.

Effects of different VLB and Doxo treatment combinations delivered with a 48-h interval between the administration of the two drugs in EAT tumour cells

The cytotoxic effects of VLB and Doxo combinations delivered with a 48-h interval

between the applications of the two drugs were also tested. In contrast to the 24-h interval between the drugs, at the 48-h interval, both treatment combinations (VLB preceding Doxo or VLB following Doxo) resulted in a significantly reduced cell survival in comparison to all other treatment groups (Figure 4).

Measurement of percentage of fluorescent cells using flow cytometer demonstrated that, in the treatment combinations with the 48-h interval, the number of Doxo positive cells was significantly lower than in the treatment with Doxo alone or with both drugs injected simultaneously (VLB + Doxo). The lower percentage of fluorescent cells observed after the treatments can be ascribed to a significant cell kill induced by these treatments (Figure 5). This is in agreement with the results observed when the animals were treated with increasing doses of Doxo alone.

In the group treated with Doxo and VLB applied with the 48-h interval between the two drug administrations, the DNA distribution measurements of the samples showed an increased G_2M compartment. The same phenomenon was observed when the animals were treated with Doxo alone or when Doxo and VLB were applied simultaneously. The DNA histograms of the samples from VLB treated animals or when VLB preceded Doxo showed less distinctive peaks and cells with very high DNA values (Figure 6). Morphological changes corresponded to the measured DNA content in the cells (Figure 7). In the experiments when VLB preceded Doxo, the effect of VLB was dominant (multinucleated cells), while in the case when Doxo preceded VLB, the dominant effect on the cell morphology was due to Doxo (enlarged cells). However, in both cases, extremely enlarged cells with potocytosis were also observed. When the drugs were given simultaneously, a certain percentage of unaffected tumour cells were observed, the rest of them displayed morphological changes that could be ascribed predominantly to Doxo action. In other treatment groups (VLB 48 h and Doxo 48 h alone), the cells displayed typical changes pertinent to the action of the drug. The cells treated with VLB alone were enlarged. In addition, irregular mitoses and multinucleated cells with potocytosis were also observed. The cells treated with Doxo alone were also enlarged having enlarged nuclei and nucleoli.

In cytological smears of all treatment groups, a high percentage of inflammatory cells was observed, which was also shown in DNA histograms, as a diploid peak.

Effect of different VLB and Doxo treatment combinations on animal survival

Survival of animals was also determined after various schedules of Doxo and VLB treatments delivered with 24-h and 48-h intervals between the injections of the drugs. Despite the significant difference in reduction of EAT tumour cell number in the ascites after the treatment with Doxo and VLB delivered with the 48-h interval between the injections, this reduction did not translate into statistically significantly prolonged animal survival. Regardless of the schedule of the treatment, median survival times did not significantly differ between the treatment groups either delivered with the 24-h interval (data not shown) or 48-h interval between the drug administration (Figure 8). However, there was a trend to prolonged survival in the group treated with Doxo and 48h later with VLB.

Discussion

The results of our study show that the combination of VLB and Doxo is schedule-dependent. The time interval between the

drug administrations, but not the sequence, seems to be crucial for the obtained effects. The interval of 48 h between the drug administrations resulted in more pronounced antitumour effectiveness compared to the 24-h interval.

In planning multidrug (combined) chemotherapy in clinical setting, attention is focused mainly on defining an optimal dosage of the drugs in a protocol with acceptable toxicity. However, for the effect of a protocol, beside dosage and toxicity, an interaction of drugs with consequent schedule-dependency could be decisive. A combination of drugs can result in a synergistic, additive or antagonistic effect. In the literature on multidrug schedules in clinical chemotherapy, there are usually only the data on the dosage of drugs and their distribution according to days in a particular cycle of chemotherapy. There are mostly no data on the sequence and exact timing of drugs.[18-22] In the reports in which authors define the sequence of drugs, there are usually no data on their exact timing and intervals between drugs [1,4,6], *i.e.* factors which could have an important influence on the effect of chemotherapy. In clinical studies comparing multidrug chemotherapy to single agent chemotherapy, a clear advantage of multidrug chemotherapy over single agents was not always shown. In a meta analysis of randomized trials in metastatic breast cancer, Fossati[23] found an advantage of multidrug chemotherapy in the response rate, but only a very modest benefit in survival of patients. Similarly, Ejlertsen[19] described a higher response rate with no benefit in survival of patients, whereas Norris and Joensuu found no advantage of combined schedules in metastatic breast cancer.[22,24] Among other factors for these conflicting results, schedule-dependency could also play a role.

Recently, there have been many reports on schedule-dependency between antracyclins and microtubule active drugs.[7-11,25] In our work, we explored the schedule-dependency of VLB combined with Doxo in Ehrlich ascites tumour cells in mice. We found that the combination was schedule-dependent. The most cytotoxic combinations were VLB injected 48 h before Doxo and the reverse order of the drug injections, Doxo injected 48 h before VLB. Both orders of sequence were equally effective and showed a statistically significant reduction of tumour cell survival compared to all other combinations (Figure 4). The combination of VLB and Doxo was not better than Doxo alone when given simultaneously or with the 24-h interval between the drugs. In contrast to our results, Zeng [10] found an antagonistic effect of Doxo and a mitotic poison Docetaxel if the two drugs were applied simultaneously or in the sequence Doxo-Docetaxel. The reverse sequence Docetaxel-Doxo delivered with an interval of 12 h between the drugs resulted in an enhanced cytotoxicity. The explanation for the antagonism of the sequence Doxo-Docetaxel backed up by flow cytometry was that Doxo produced a block in G_2 phase of the cell cycle and thus prevented the mitotic arrest by Docetaxel.[10] Similarly, the best inhibition of the tumour by the schedule Docetaxel interval 12 h Doxo was reported by To *et. al.* in Ehrlich ascites cell tumours bearing mice.[9] In contrast to these results, Zoli found a synergistic and not antagonistic effect of the sequence Doxo and another microtubule stabilizing agent Paclitaxel.[11] The results of schedule-dependency studies are influenced by many factors, such as the dosage, time of exposure to the drugs and the intervals between the injections of drugs. In addition, the results could depend on the tumour model as different cell lines can respond with different cell kinetic changes to the same drug.[26]

Flow cytometric study showed two distinctive patterns of DNA distribution after

the treatment with VLB or Doxo. The latter drug produced a block of cells in the G_2M compartment as shown previously[27,28], whereas after VLB, a wide scatter of DNA values with less distinctive peaks in comparison with Doxo were observed. When both drugs were given simultaneously, the effect of Doxo prevailed, whereas in combinations delivered with an interval of 24 or 48 h between the drugs, the pattern of the first drug prevailed (Figure 6). Similarly, cytomorphologic studies showed two distinctive patterns of changes after VLB or Doxo. After Doxo, the tumour cells were enlarged with enlarged both nuclei and nucleoli. Mitoses in the smears were very rare, which means that the signals appearing in the DNA histograms in the G_2M compartment represent nuclei blocked in the G_2 phase of the cell cycle. In contrast, after VLB, cells with irregular mitoses as well as multinucleated cells as a consequence of unaccomplished cell division were observed. In the DNA histograms, scattered, very high DNA values depassing the G_2M peak of the tumour were found, correspondingly. VLB exerts its action at least in part by binding to tubulin and disturbs the function of microtubules, necessary for the formation of mitotic spindle. After discovery of VLB, it was believed that its primary action is depolymerization of microtubules. Only recently it was discovered that, at low doses, VLB stabilizes microtubule dynamics and blocks or slows down the mitosis by acting on microtubule dynamics and not by depolymerization as at high doses.[29,30] The consequence of impaired function of mitotic spindle is the mitotic arrest and inhibition of cell proliferation. However, as antiproliferative activity of VLB does not correlate well with binding to tubulin, there must be other targets for VLB such as RNA, DNA and lipid biosynthesis.[29]

Better results of the combinations of VLB and Doxo applied with the 48-h intervals over the drugs applied simultaneously can only partly be explained by DNA measurements and cell morphology studies. The block in G_2 could prevent cells entering mitosis, which is the part of the cell cycle where VLB can exert its maximal effect. We could speculate that the sequence of Doxo and VLB could therefore be self-limiting. Such an explanation could be valid for the simultaneous application of both drugs or when the time interval was 24 h where the effect was not better than that of Doxo alone. On the other hand, such an assumption does not explain the best results obtained after administering the drugs in 48-h intervals between them irrespective of their sequence. Our DNA measurements in this experiment showed that the block of the cells in G_2 was still present 48 h and even 72 h after the application of Doxo, yet the tumour cell toxicity was enhanced with the combination of Doxo and VLB provided the drugs were delivered with the 48-h interval between the drug administrations. Moreover, not the sequence of drugs, but the time interval between them was crucial for obtaining better effect. According to our results, the explanation could be that the action of either of the drugs, Doxo or VLB, needs 48 h to make the tumour cells either prone to the action of the other drug or to trigger a cell death pathway.

Our second objective was to explore whether pretreatment with VLB will increase the accumulation of Doxo in tumour cells. In our previous preclinical study, we showed that VLB increased cell membrane fluidity.[16] Pretreatment with VLB increased the uptake of cisplatin into the tumour cells, which led to an increased antitumour effectiveness of cisplatin.[14] An increase antitumour effectiveness was also demonstrated for bleomycin applied after VLB.[15] In the present study, the pretreatment with VLB did not result either in an increased accumulation of Doxo or in the increased

antitumour effectiveness compared to the treatment with reverse order of the drugs. There are reports that the effect of Doxo is not dependent solely on entering the cell and binding to DNA, but also on binding to the cell membrane, which is very important for inducing the cell death.[31-33] Therefore, we assumed that the cytotoxic effect of the combination of Doxo and VLB applied with the 48-h interval between the drug administration might be the result of the effects on DNA, cell membrane events and transmembrane signalling.[31-34] Another explanation for better results of the schedule with 48-h interval could be a repopulation of tumour cells which might occur after that interval and the elimination of the repopulated cells by the second drug.

The fact that no drug combination resulted in a prolonged survival of animals (Figure 9) is not surprising. We deliberately used low doses of VLB and Doxo with the aim to demonstrate a possible interaction of the combinations. Even after the best combinations with 48-h interval between the drug injections, there were still some unaffected tumour cells in the specimens. In view of rapid repopulation of tumour cells in this fast growing tumour model, the difference in animal survival could not be expected after only one cycle of chemotherapy.

In conclusion, the combination of VLB and Doxo is schedule dependent. It seems that for the effect of treatment the time interval between the drug administrations, but not the sequence of drugs, is crucial. Pretreatment with VLB does not increase the accumulation of Doxo in EAT tumour cells. When translating the results of preclinical studies to clinical setting, we need to be cautious, since different tumour models used can yield controversial results. Nevertheless, the data from preclinical studies should be taken into consideration when planning combined treatment schedules in clinical situation.

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Cysteine cathepsins, stefins and extracellular matrix degradation during invasion of transformed human breast cell lines

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Background. *Human breast cellular model, comprising four cell lines originating from spontaneously immortalized human breast epithelial MCF10A cell line, its c-Ha-ras transfectant, MCF10AT, and two tumourigenic derivatives, cultured from two sequential mouse xenographs, MCF10AT-Ca1a and MCF10AT-Ca1d, were used to compare the relative protein concentration of cathepsins and stefins in single cells.*

Methods. *The relative protein concentration of cathepsins and stefins in single cells was analysed by confocal microscopy, and compared to their protein expression in cell homogenates.*

Results. *The most invasive, MCF10AT cell line contained several fold higher protein concentration of cathepsin B and increased levels of stefins, but similar levels of cathepsin L, compared with the parental MCF10A cells. This was associated with five fold higher endocytosis of Matrigel-DQ-collagen IV (DQC) and a simultaneous increase in signal overlap between DQC and cathepsin L as well as DQC and stefin B, but a decrease in that of DQC and cathepsin B overlap in the MCF10AT cells. Simultaneously, increased signal overlaps between both cathepsins and between cathepsins-stefins pairs, were observed in this cell line.*

Conclusions. *These results suggest that the increased collagen endocytosis and degradation in the invasive phenotype significantly affect also the subcellular localization of cysteine cathepsins and stefins. Based on these and the reports of other authors, we hypothesize that the intracellular degradation may also be associated with cathepsin L, whereas cathepsin B in the ras transformed breast cells is involved in both, the intracellular and pericellular degradation of extracellular matrix during cell migration and invasion.*

Key words: breast neoplasms; tumor cells, cultured; neoplasms invasiveness; cathepsins; extracellular matrix

Introduction

Human genome is known to contain 11 related, but distinct cysteine proteases belonging to papain family C1A, cathepsins (Cats) B, L, H, S, K, F, V, X, W, O and C.[1] Early studies of CatB and CatL have shown

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their wide tissue distribution. They are mostly localized to lysosomes, where they can reach up to 1 mM concentration.[2] In the tumour cell models, the increased secretion of the pro-enzyme and the mature enzyme forms of CatB[3,4] and CatL[5] after the oncogenic transformation has been reported. Besides other proteases, CatB[6] and CatL[7] can directly degrade components of the extracellular matrix, although under slightly different conditions. It has been shown that CatL activation may be accelerated by certain ECM components[8], such as proteoclycans.[9] Cathepsins may also be involved in the proteolytic cascade[10], in which they activate other proteases. In breast carcinoma, altered expression of lysosomal CatB and CatD and altered subcellular trafficking were initially demonstrated by Sameni *et al.*[11] and later confirmed by these and other authors (reviewed in[12]). The interest in the mechanisms of CatB and CatL regulation and activation increased when several clinical studies have shown elevated mRNA, protein and activity levels of CatB and CatL in malignant breast tumour tissues and demonstrated their potential for the prognosis of the disease.[13-16] The activities of cysteine cathepsins are regulated by their endogenous inhibitors, a large superfamily of cystatins. The stefins (St) family comprise the intracellular inhibitors, of which StA and StB were also found to be altered in tumours and sera of cancer patients.[17-20] The structural features of inhibitors and their tight complexes with cathepsins were revisited by Turk and Gunčar.[21] Presumably, their cytosolic location should guard the subcellular structures from the accidental release of active lysosomal cysteine cathepsins, but their intracellular interactions with their target proteases are still not well understood.

To establish whether CatB and CatL are associated with an invasive cell phenotype and with the ability to form tumours after the injection at a secondary site (tumourigenicity), we have used a cell model of four breast cell lines, originating from parental MCF10A cell, which is described in details in Materials and methods below. In this model, we found that the *in vitro* invasiveness did not correlate with cell ability to form malignant tumours in mice, the most invasive cell line being the MCF10AT and selective synthetic inhibitors of CatB and CatL impaired the *in vitro* invasion of these cells.[22] In the present study, we focus on the expression and the localization of CatB and CatL and their inhibitors StA and StB in the transformed breast cells during the invasion through Matrigel-collagen matrix. The aims of this study were: (a) to determine the relative abundance of cathepsins and stefins in different breast cells during their invasion into Matrigel-collagen matrix using confocal microscopy in comparison with their expression levels in cell homogenates, (b) to develop the software tools for faster, cost-effective, automatic and more objective single-cell image stack analysis, including analysis of signal overlapping of different antigens and (c) to examine the overlapping signals of cathepsins and stefins inside the tumour cells, along with the degraded endocytosed collagen type IV (DQ-collagen IV) in the parental MCF10A, and invasive MCF10AT cell lines.

Materials and methods

Cell lines

We used a model of four epithelial breast cell lines derived from spontaneously immortalized cells of a fibrocystic breast patient. The parental cell line was the immortalized diploid cell line MCF10A.[23] The MCF10AT line is MCF10A transfected with c-Ha-*ras* oncogene[24,25] and has an acquired ability to grow in immunodeficient mice. MCF10AT-Ca1a and MCF10AT-Ca1d, obtained by

multiple passages of MCF10AT cells in nude mice, are fully malignant. MCF10AT-Ca1a cells mostly produce undifferentiated carcinoma and MCF10AT-Ca1d cells form heterogeneous carcinomas.[26] These lines were originated at Barbara Ann Karamanos Cancer Institute (Detroit), and kindly provided by Prof. Bonnie Sloane, Department of Pharmacology, WSU, Detroit MI, USA. Cell lines were grown as described previously.[22]

Preparation of cell lysates and enzyme-linked immunosorbent assay (ELISA)

The cells were scraped and pelleted by centrifugation at 150g for 5min. They were homogenized by sequential freezing in liquid nitrogen and thawing at 37ºC (3 X) in 50mM Tris buffer, pH 6.9, containing 0.05% (v/v) Brij 35, 0.5mM DTT (dithiothreitol), 5mM EDTA, 0.5mM PMSF (paramethylsulphonyl fluoride) and 10mM pepstatin A. The lysates were centrifuged at 12,000g for 15min and the supernatants stored at –20°C.

ELISA kits for human CatB, CatL, StA and StB were obtained from Krka d.d., Slovenia and performed as suggested by the manufacturer. Purified human CatB, CatL, StA and StB were used as the standards. For CatB, rabbit polyclonal anti-CatB antibodies (IgG) and sheep anti-CatB antibodies (horseradish peroxidase-conjugated) were used as the capture and detection antibodies, respectively. For CatL, polyclonal sheep anti-CatL, for StA, monoclonal mouse anti-StA, and for StB, monoclonal mouse anti-StB antibodies were used as the capture and the detection antibodies. Total protein concentrations were determined using Bradford assay (Bio-Rad, USA). Mean values of at least three independent measurements and standard errors of the mean (SEM) were calculated. Statistical significance was determined with t-test and $p < 0.05$ was considered significant.

Confocal microscopy

Each of 8 wells on a Chamber Slide™ (Lab Tec®, Nunc Inc., USA) was pre-coated with 150 μl of fibronectin (16.7 μg/ml, 2.5 μg per well, Sigma) and coated with 200 μl of Matrigel (1 mg/ml, Becton Dickinson, USA), with added 0.2%, or 1% fluorescently labelled DQ-collagen IV, DQC (Molecular Probes, USA). 200,000 cells in 100 μl of medium were seeded and grown for another 24 h. Then the cells were washed with PBS and fixed with 200 μl of 3.7% formaldehyde for 30 min at 37 C in 5% CO_2. After washing with PBS, cells were permeabilized for 5 min with 150 μl of 0.2% Triton X-100 (Sigma). Polyclonal and monoclonal antibodies were tested with comparable intensity of labelling and herein mouse clones of the primary antibodies for cathepsins and stefins were used (all by Krka d.d., Slovenia) at 2 mg/ml final concentration. Secondary antibodies, excited at various wavelengths, AlexaFlour 488 rabbit anti-mouse IgG, AlexaFlour 546 goat anti-mouse IgG, and AlexaFlour 633 rabbit anti-mouse IgG, were then applied according to the instructions of the supplier (Molecular Probes, USA). Samples were imaged using laser scanning confocal microscope Leica TCS SP2 at the three channels with excitation wavelengths 488 nm, 546 nm and 633 nm. Images were recorded at 1000× enlargement in series of fifty to eighty 512×512 pixel images (size of a pixel in x and y directions app. 50-300 nm) at 150-1000 nm distance in the z-axis. Each cell (cell group), *e.g.* each stack of images, was then analyzed with Image software (available on the internet and developed by Wayne Rasband, wayne@codon.nih.gov). Software analysis, customised plug-ins, were developed in our laboratory specifically for this purpose. Due to the space limitations these plug-ins are not explained in detail but are available upon request.

The first plug-in was developed to detect edges of the cells. The selected regions of

interest were then transformed by another plug-in into a mask, used to separate possible multiple cells. These masks were then used for quantification, with pixels outside the mask taken as zero signals. The algorithm applied to the mask calculates the volume of the cell (in actual units, e.g. μm^3), absolute and average values of the signal in each of the three channels (488 nm, 546 nm and 633 nm) and allows signal-overlap measurements of the three channels using a specifically derived formula.[27] Another plug-in detects background (noise) structures of non-specific shape and deletes them. Although some noise still has to be omitted manually by checking each slice, our method is much faster, and more precise than manual selection of cell edges.

Overlapping signals are the closest this method can get to actually monitor the antigens' subcellular co-localizations. As the size of the cell in pixels (radius between about 50 and 200 pixels) is not large enough for precise separation and localization of the organelles, the signal overlap relates to the localization of the antigens to the same area of the cell, but not to the co-localization to the same subcellular organelle. This signal-overlap was determined by calculating "overlap index" for each pixel of the image (500 × 500 × 50-80 pixels). The index was calculated for each pair of the channels separately (488 nm – 546 nm, 488 nm – 633 nm, 546 nm – 633 nm). The index took into account the relative signal intensity (0-256) of each pixel compared to the background of the individual slice (generally between 10 and 20). These relative intensities were therefore generally larger than one but not higher than about 20. Finally, relative signal intensities of the two signals (e.g. at 488 nm and 546 nm) were multiplied so that only in the cases where signals in both pixels were strong, the index could reach high enough values to be taken into consideration for calculation. The average overlap index across the cell and at the wavelength pairs was calculated by the appropriate statistical analyses, using up to 50 single-cell measurements for each category, *e.g.* images with different combinations of labelled Cats B and L, Sts A and B and degraded DQC, respectively.[27]

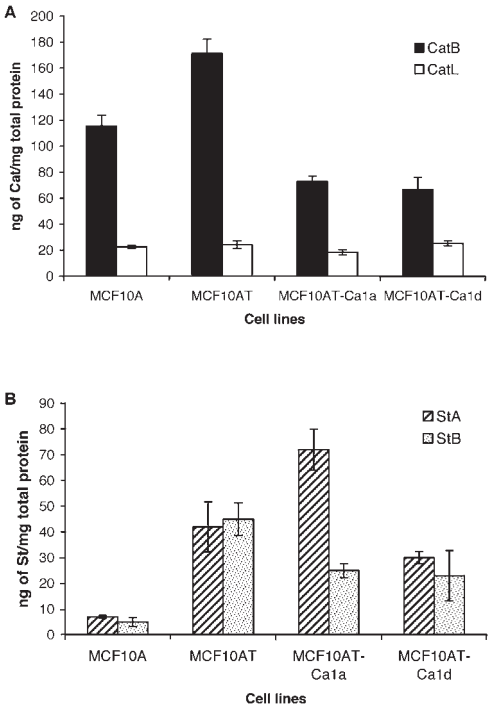

Figure 1. *Protein expression of CatB and CatL, StA and StB in the lysates of human breast epithelial cell lines, MCF10A, MCF10AT, MCF10AT-Ca1a and MCF10AT-Ca1d.*
Cells were grown on Matrigel, homogenized and protein expressions of cathepsins and stefins were determined by ELISAs as described in Materials and methods. Mean values from three independent experiments are presented. Error bars depict SEM. The statistical significance was determined by t-test and $p < 0.05$ was considered significant. The cell lines are listed according to their increased tumourigenicity.
(a) CatB protein concentration was the highest in MCF10AT cells ($p = 0.003$) and significantly lower in MCF10AT-Ca1a ($p = 0.002$) and MCF10AT-Ca1d cell lines ($p = 0.004$), compared to MCF10A cells. CatL expression was similar in all four cell lines.
(b) StA and StB were both significantly increased in MCF10AT, MCF10AT-Ca1a, and MCF10AT-Ca1d cells (all $p<0.005$) compared to the parental line.

Results

Cathepsins and stefins protein expression in cell cultures by ELISA

As previously demonstrated, the MCF10AT, obtained after *ras* transfection of the parental MCF10A cell line, was the most invasive, whereas the two cell lines, MCF10AT-Ca1a and MCF10AT-Ca1d, which were obtained from xenographts of the MCF10AT cell line, although having higher tumourigenicity than the parental cell line, are less invasive in *in vitro* Matrigel assays.[22-27] CatB protein expression was the highest in MCF10AT ($p = 0.003$), and significantly lower in MCF10AT-Ca1a ($p = 0.002$) and MCF10AT-Ca1d cell lines ($p = 0.004$), compared to MCF10A cells (Figure 1a). CatL expressions were similar in all four cell lines.

StA and StB were both significantly increased in MCF10AT, MCF10AT-Ca1a, and MCF10AT-Ca1d cells (all $p<0.005$) compared to the parental line (Figure 1b). Noteworthy, StB expression was the highest in MCF10AT, similar as was that of CatB. The relative expression of these antigens was similar as observed previously,[22] when these cells were grown on the plastic surface, although on the Matrigel, all protein levels are about two fold lower compared to the plastic surface. Noteworthy, the molar ratios calculations (between cathepsins and stefins) showed that there was about 60-90 fold overexpression of cathepsins in MCF10A, and slightly decreased in the malignant cells lines.

Analysis of cathepsins, stefins and the Matrigel-collagen by confocal microscopy

A representative example of immunohistochemical labelling in a model of the four lines is shown on Figure 2. The quantitative fluorescence analysis of single cell images by confocal microscopy parallels the data in cell lysates. Quantification of CatB in MCF10A and MCF10AT cells by confocal microscopy showed its significantly higher expression in the MCF10AT than in the parental cells. The increased pericellular distribution of CatB was observed in MCF10AT, confirming the previous findings.[11,28] In MCF10AT-Ca1a and MCF10AT-Ca1d cells, spreading of CatB immunostaining over the whole cell was observed. CatL distribution was not significantly changed in these cells compared to MCF10A cells. The expression of stefins was markedly elevated, particularly in tumourigenic cell lines, compared to MCF10A cells.

The last panel represents degraded DQC (green) in the four cell lines, grown on Matrigel, enriched with DQC. Slight, pericellular degradation of DQC was observed in the parental cells. In contrast, the intracellular degradation of DQC in MCF10AT was about 5-times higher than in MCF10A cells and both, extracellular and pericellular degradation of DQC was observed. The intracellular degradation of DQC was also abundant in the tumourigenic cells in spite of the fact that the latter are less invasive *in vitro.*[22] Taken together, these results show that the parental cell line contained the least of endocytosed matrix, least dispersion of cathepsins staining and low levels of both stefins.

Subcellular distribution and co-localization of antigens

The 3-D analysis was performed on a sample size a few hundred single cells. These were imaged in over 100 stacks of 50-80 slices with the size of 512 × 512 pixels at three different wavelengths of the excitation laser, as described in Material and methods. For reasons of clarity, we will present the process of the confocal image analysis using one slice of a single stack. Figure 3a presents a raw image of the MCF10AT cell labelled with DQC (488nm channel – left),

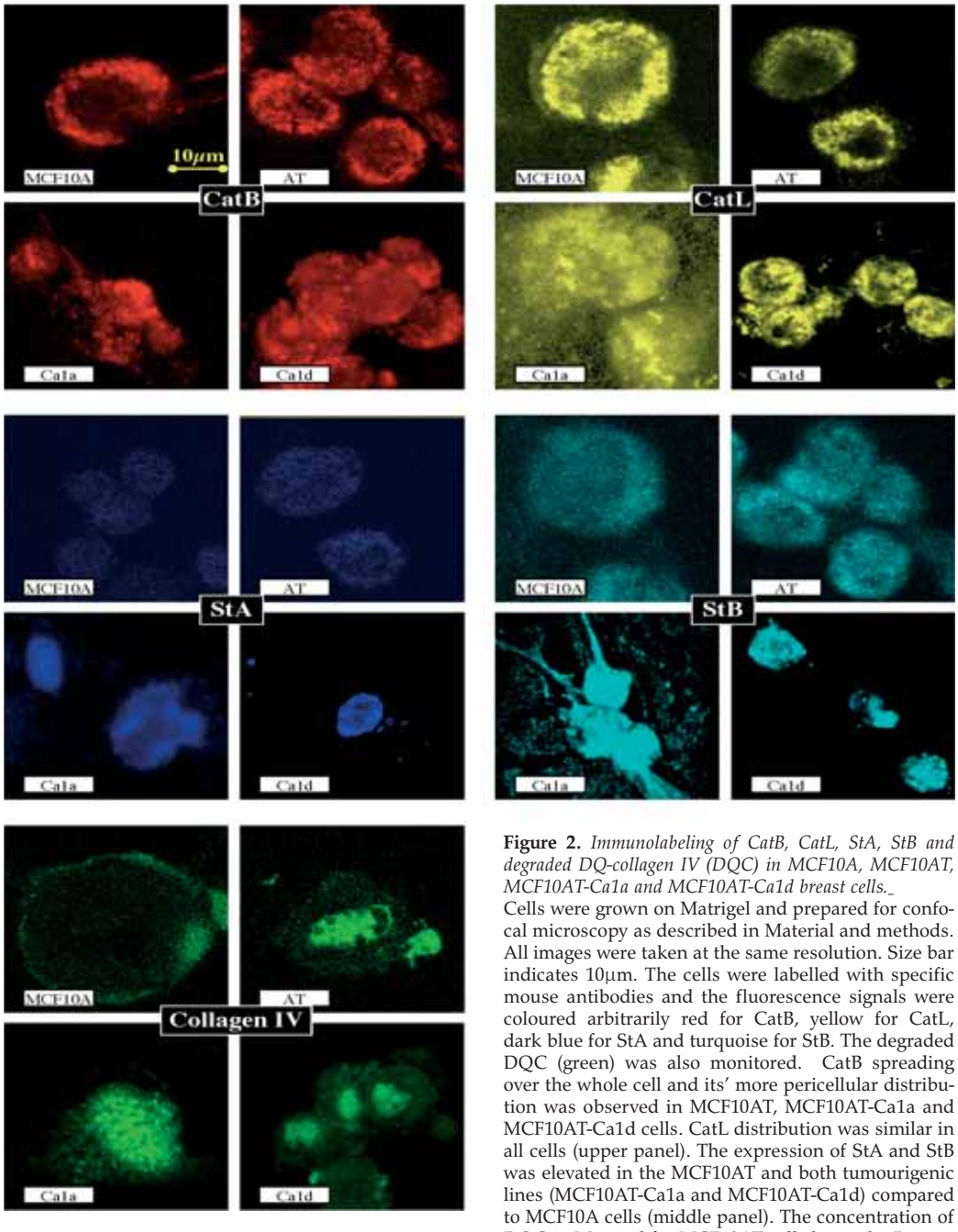

Figure 2. *Immunolabeling of CatB, CatL, StA, StB and degraded DQ-collagen IV (DQC) in MCF10A, MCF10AT, MCF10AT-Ca1a and MCF10AT-Ca1d breast cells.*
Cells were grown on Matrigel and prepared for confocal microscopy as described in Material and methods. All images were taken at the same resolution. Size bar indicates 10μm. The cells were labelled with specific mouse antibodies and the fluorescence signals were coloured arbitrarily red for CatB, yellow for CatL, dark blue for StA and turquoise for StB. The degraded DQC (green) was also monitored. CatB spreading over the whole cell and its' more pericellular distribution was observed in MCF10AT, MCF10AT-Ca1a and MCF10AT-Ca1d cells. CatL distribution was similar in all cells (upper panel). The expression of StA and StB was elevated in the MCF10AT and both tumourigenic lines (MCF10AT-Ca1a and MCF10AT-Ca1d) compared to MCF10A cells (middle panel). The concentration of DQC in Matrigel for MCF10AT cells has to be 5-times lower than in other cell lines, otherwise the signal was too strong for imaging. Intracellularly degraded DQC was expressed at a higher level in MCF10AT, MCF10AT-Ca1a and MCF10AT-Ca1d cells compared to the parental cells.

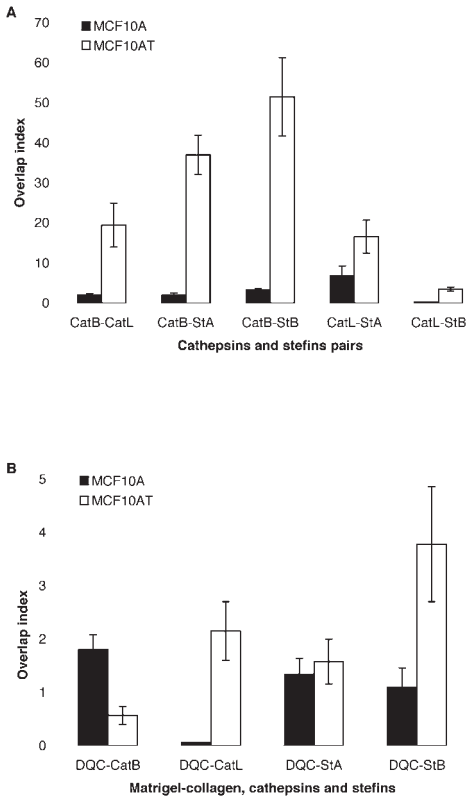

Figure 4. *Signal-overlap of cathepsins, stefins and DQ-collagen in MCF10A and MCF10AT cells.*
The overlap index was calculated as described in Materials and methods. Error bars depict SEM. Note the different scales of panels A and B. **(a)** Overlap indexes between cathepsins and stefins were measured in all pairs. The signal overlap was higher in MCF10AT cells than in the MCF10A cells. This increase in the signal overlap was highly significant between CatB and both stefins, and between CatL and StB ($p = 0.0001$), less so between CatB and CatL ($p = 0.0065$), and barely significant between CatL and StA ($p = 0.049$). In invasive MCF10AT cells, CatB-CatL overlapping was relatively low compared with the overlap indexes between CatB and stefins. **(b)** Overlap indexes between DQC and CatB, CatL, StA, and StB showed a significant increase in the signal overlap observed in MCF10AT compared to MCF10A cells between DQC and CatL ($p = 0.0002$) and DQC and StB ($p = 0.0155$). In contrast, the overlap index between DQC and CatB was significantly lower ($p = 0.0018$) in MCF10AT cells. That between DQC and StA was similar in both cell lines.

the antibodies against CatL (546nm – middle) and the antibodies against StB (633nm – right). To these raw images we applied the first and most extensive plug-in, that used an algorithm designed to detect whether or not there is a significant gradient of signal strength, which dubbed an edge at each individual point. The plug-in also detected the angle at which this edge was going through the pixel in question. To achieve this, a roster of 9 × 9 pixels surrounding the pixel in question was analysed. If adjacent pixels had edges running in the same direction (angles not differing by more than a preset number, e.g. 5° or 10°), an edge was drawn on the resulting image (Figure 3b). In Figure 3c, the results of the second plug-in are shown. It was designed to check for the size and shape of the resulting closed shapes and to fill the shapes that were meeting the criteria of size and circularity. The next plug-in (Figure 3d) combined all three channels into one (Figure 3d middle). The red line was manually drawn after reviewing single slices (Figure 3d left) and comparing it to a composite image throughout the depth of all 55 slices (Figure 3d right).

Figure 4a shows the signal overlap between both cathepsins and between cathepsins and stefins. In MCF10AT cells, CatB overlapped better with either of the stefins than CatL did. Consistently a higher signal overlap observed in MCF10AT cells than in the paternal MCF10A cells, may be also due to the increased immunostaining of both cathepsins and stefins in the former cell line. Figure 4b presents the signal overlap indexes between the degraded DQC (Matrigel) and cathepsins and stefins in the invasive MCF10AT compared with the benign parental cell line. A significant increase in the signal overlap was observed in MCF10AT compared to MCF10A cells between DQC and CatL ($p = 0.0002$) and DQC and StB ($p = 0.0155$). In contrast, the

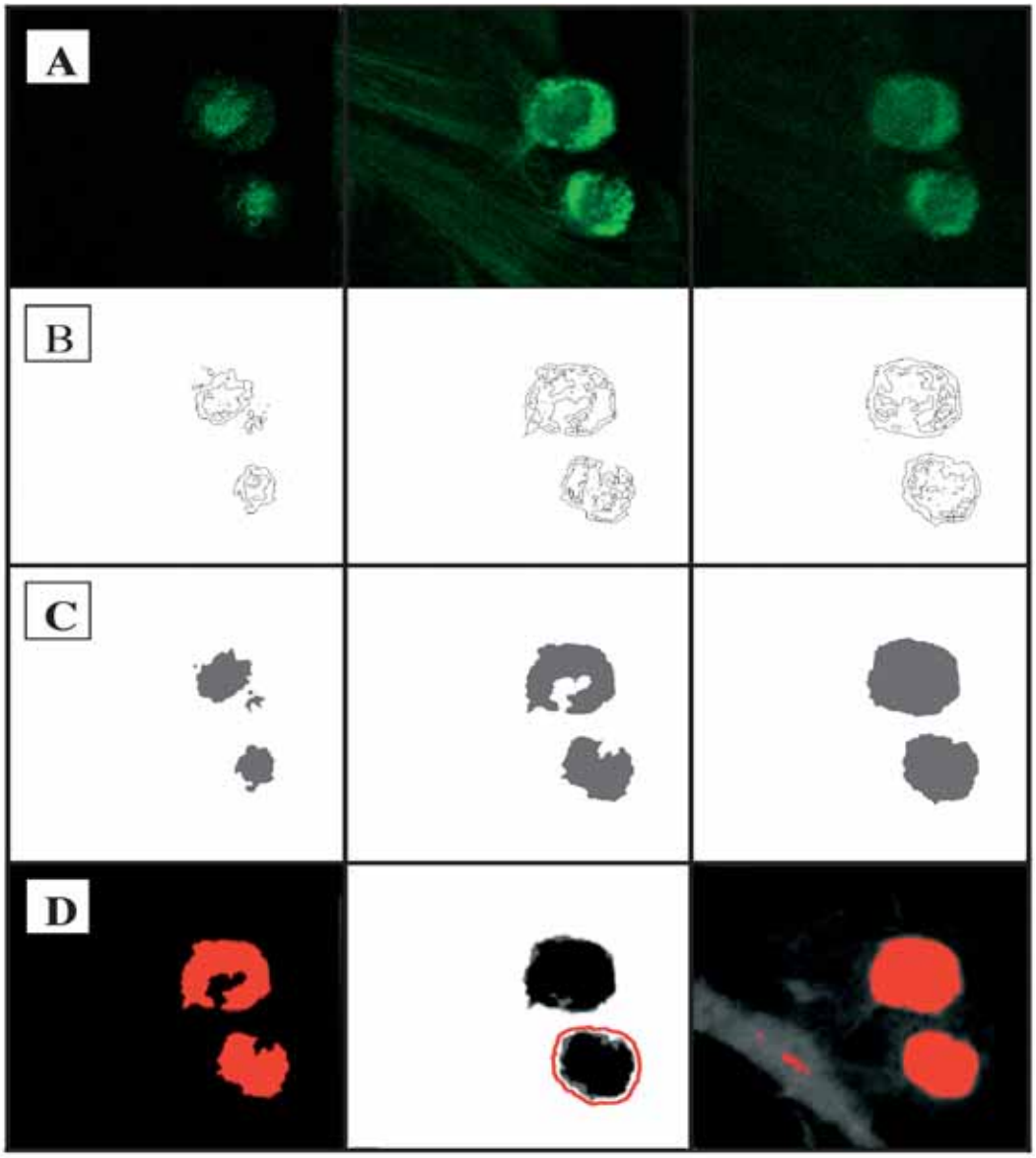

Figure 3. *The development of software tools for the quantitative image analysis of confocal images.*
A single slice of a MCF10AT cell is presented**.** The software plug-ins are described in Materials and methods. The stack of images can be browsed using a slide bar on the bottom to view any slice of the stack at any chosen time. **(a)** The un-edited images of a single slice of MCF10AT cell at three different wavelengths: left – 488nm, DQ-collagen IV; middle – 546nm, CatL, right – 633nm StB, as derived from the confocal microscope. The green color was chosen arbitrarily. **(b)** The same slice after the plug-in for edge detection. The plug-in automatically detects cell borders and intracellular margins based on the changes in the intensity of the signal that meet predefined criteria. **(c)** The slice after the plug-in that fills the encircled areas and removes the areas that are too small or do not meet the defined shape criteria. The removing process can be best observed when panels (b) and (c) of the left image are compared. **(d)** Final (manual) processing of the slice. Left: The three areas calculated in (c) were merged into one that represents the total area of interest in the slice. Middle: The area representing a single cell was selected manually (red line). Right: The view through the whole stack (50-80 slices) allowed the easier overview of the cell size and position. Some structures shown on this view clearly do not represent cells and these were removed by the plug-ins.

overlap index between DQC and CatB was significantly lower ($p = 0.0018$) in MCF10AT cells. That between DQC and StA was similar in both cell lines. Similarly, the overlap index between the DQC and StB was significantly increased in MCF10AT cells, whereas that of stefin A was similar as in MCF10A cells.

Discussion

The present work is the continuation of the study on lysosomal cathepsins and their inhibitors in a panel of four human breast epithelial cell lines, which represents a cellular model for the development of malignant phenotypes.[22] We examined the expression of cysteine cathepsins and their inhibitors at protein levels in cell lysates on one hand, and on the other hand by confocal microscopic imaging, which may give an alternative insight into the proteolysis associated with the invasion into collagen matrix. For the latter we developed a novel software programme for the partially automatic analysis of confocal images by using multiple imaging channels to monitor and quantitate the proteins along with the degradation of DQC. Imaging data confirmed the measurements in cell lysates, showing increased levels of CatB, but not CatL protein in the most invasive, *ras* transfected cells. CatB was lower in the two less invasive, but more tumourigenic cell lines and in the paternal cells, whereas CatL expression was similar in all four cell lines. These results indicated that the *ras* transfection of the breast epithelial cells affected specifically CatB expression and mostly its trafficking,[11,28] but not that of CatL. This is different as recently reported by Collette *et al*. for mouse fibroblasts and rat ovarian epithelial cells, where *ras* signalling pathways resulted in markedly up-regulated CatL, but not CatB.[5] The authors also emphasized that *ras* signalling is cell type specific and may affect lysosomal cathepsins in different ways. Indeed, in our transformed breast cells, CatL and CatB were differentially affected by the *ras* oncogene transfection and were obviously independently regulated. A functional role for *ras* in cathepsin B trafficking and redistribution to cell surface has also been observed in melanoma, osteoclasts and human colorectal carcinoma (sumarized in ref [12]), similar as observed in this study. We also reported on the independent regulation of both cathepsins in another panel of breast cancer cell lines[29-31] and same notion was deduced from the clinical studies.[13,15,16,32]

It has been suggested that a misbalance between cathepsins and their inhibitors contributes to the progression of tumors.[16-18] In this study, both stefins were upregulated in the more invasive as well as in the tumourigenic cells. From the calculated molar ratios between individual cathepsins and stefins, being much higher than equimolar, we may conclude that the concentration of these two inhibitors may not be sufficient to inhibit these two cysteine cathepsins. However, in another panel of breast cancer cells lines, obtained from breast cancer patients with different genetic alterations, we found an inverse correlation between the invasiveness and stefins' expression.[30] In this and other studies on expression of stefins in cancer progression, the findings are not conclusive: lower, similar or higher levels of stefins were found in tumour tissue homogenates in the clinical studies of breast and prostate carcinoma,[32,33] suggesting the complex regulation of stefins in tumours progression.

In this study, we observed a partial signal overlap between cathepsins and stefins, indicating that they are to some extent colocalized to the same cellular area, but not necessarily to the same cellular organelles. This supports the data from the first at-

tempt to localize the cathepsins and stefins by confocal microscopy, revealing their differential localization in human embryonic liver and hepatoma cells.[34] Noteworthy, CatB and StB co-localization seems to be the highest in the invasive cells, whereas CatL co-localization with stefins was relatively lower in the invasive cells. This may result in higher CatL efficacy in the intracellular collagen degradation, which certainly also depends on the local access of stefins to bind to CatL.

Collagen turnover is critical to tumour expansion and several previous studies addressed the questions of the subcellular sites of its degradation. Sameni *et al.*[35] showed that collagen degradation was predominantly pericellular in BT20, and mostly intracellular, localized to lysosomes, in BT549, both breast cancer cell lines. They have also demonstrated that proteolysis is pericellular in breast carcinoma spheroids and pericellular, as well as intracellular, in the colon carcinoma spheroids, with the stroma and inflammatory cells contributing to the degradation of collagen.[36] In prostate carcinoma cells, the DQ-collagen I and DQ-collagen IV degradation was reduced by inhibitors of matrix metallo, serine and cysteine proteases,[37] suggesting a complex interplay among these proteases in matrix degradation. In the MCF10A-neoT cells, Premzl *et al.*[28] clearly defined a co-localization of extracellular and intracellular fraction of CatB, presumably responsible for both, the pericellular and intralysosomal DQ-collagen IV degradation, respectively. The authors also reported on the major role of cathepsin B activity in the intracellular substratum degradation during capillary–like tube formation of endothelial cells grown on Matrigel by differential inhibition of Z-Arg-Arg-cresyl violet degradation by the inhibitor CA-074Me and not by the non-methylated form CA-074, under the conditions permisive for cell penetration.[38] However, Montaser *et al.*[39] demonstrated that CA-074Me inactivates both CatB and CatL within living murine fibroblasts, whereas CA-074 is really inhibiting CatB selectively and therefore all previous data do not exclude the involvement of CatL in the substratum degradation. However, to our knowledge, there is no direct evidence for CatL involvement in the intracellular substratum degradation, for example by using highly selective inhibitors and/or selective substrates for CatL that would easily penetrate the living cells.

The intracellular localizations of collagen may be explained by findings of Kjøller *et al.*,[40] who reported that a glycoprotein, urokinase plasminogen activator receptor-associated protein (uPARAP/Endo180) was responsible for import and lysosomal delivery of extracellular collagen IV and that its degradation was impaired in the presence of the cysteine protease inhibitors. Genetic ablation of uPARAP/Endo180 impaired collagen turnover during mammary carcinoma progression.[41] Montcourrier *et al.*[42] found that another lysosomal cathepsins, CatD, is co-localized to and responsible for the degradation of the extracellular matrix in large acidic vesicles in the breast cancer cells, and speculated that increased intracellular substratum degradation is not only assisting tumour cell invasion, but is also important to provide tumour cells with amino acid reservoir, necessary for their the increased metabolic activity and protein synthesis. In line with this hypothesis is our finding that the intracellular degradation of collagen was not only elevated in the invasive cell type, but was actually elevated in all cancer cells lines, compared to the parental cell line, regardless of their invasive potential.

The presented confocal microscopy data are an attempt to quantitate the signal overlap of the key players of the intracellular cysteine dependent proteolysis and their

functions. A high signal overlap of CatL (but not that of CatB) with the intracellularly degraded collagen (despite being present in about five times lower concentrations than CatB) indicates that CatL may be more important for the intracellular degradation of collagen type IV. A high signal overlap index between the stefins and degraded DQC suggests that stefins are in the proximity of the cathepsins-collagen complexes and may affect cathepsins activities. However, to confirm their functionality, *in situ* activity and the co-localization of enzymes and inhibitors, additional experiments by simultaneous organelle-specific labelling are needed.

In conclusion, we were able to show that the semi-quantitative analysis of cellular levels of cathepsins and stefins by confocal images in single cell gives complementary information to the measurements in the cell lysates. Confocal microscopy and the developed software for the single-cell image stack analysis supported the biochemical analysis, demonstrating an increased labelling of CatB, StA and StB in the invasive MCF10AT compared with the parental MCF10A cells, whereas CatL was expressed equally. Much higher levels of endocytosed, degraded DQC in the invasive than in the parental line, and a simultaneous increase in the signal overlap of collagen and CatL, suggests possible involvement of CatL in the intracellular degradation of the substratum, complementary to that of CatB, what has to be confirmed in future experiments.

Acknowledgments

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Radiol Oncol 2006; 40(4): 205-9.

Intrakapsularni in paraartikularni hondrom kolena: Prikaz štirih primerov in pregled literature

Samardziski M, Foteva M, Adamov A, Zafiroski G

Izhodišča. Intrakapsularni in paraartikularni hondrom sta redka oblika hondroma, ki se pojavlja izven skeletnega tkiva. Zraste iz ovojnice in/ali obsklepnega vezivnega tkiva velikih sklepov (največkrat kolena) zaradi hrustančne metaplazije. S časom tumorji osificirajo in od tod tudi njihovo drugo poimenovanje paraartikularni osteohondrom. Po mnenju Jaffeja gre za isto entiteto ne glede na stopnjo osifikacije.

Prikaz primerov. Poročamo o štirih primerih paraartikularnega hondroma kolena. Pri kliničnem pregledu smo ugotovili počasi rastočo zatrdlino na kolenu in zmerno bolečino. Pri vseh primerih sta rentgensko slikanje in računalniška tomografija pokazali mehkotkivni tumor z različno stopnjo osifikacije; histološka preiskava pa prisotnost zrelega hialinega in vezivnega hrustanca.

Zaključki. Te benigne tumorje smo diagnosticirali s primerjavo kliničnih, radioloških in histoloških značilnosti. Vse bolnike smo radikalno operirali, kar je najprimernejši način zdravljenja.

Radiol Oncol 2006; 40(4): 211-5.

Uporaba Dopplerskega ultrazvoka pri ugotavljanju in sledenju mišične rupture in arteriovenozne fistule na stegnu 12-letnega dečka

Pavčec Z, Žokalj I, Saghir H, Pal A, Roić G

Izhodišča. V članku avtorji na kliničnem primeru prikazujejo natančnost neinvazivnih slikovnih metod, zlasti Dopplerskega ultrazvoka pri ocenjevanju mišične poškodbe in obskeletnih žilnih nepravilnostih v mehkih tkivih.

Prikaz primera. Obravnavali so 12-letnega dečka s poškodbo desne štiriglave stegenjske mišice, kjer je nasta mišična ruptura. Po operaciji so z običajnim 2-dimenzionalnim ultrazvokom videli ponavljajoči hematom. Z ultrazvočno preiskavo so pravtako ugotovili hipervaskularizirano področje in posumili na žilno nepravilnost (angiom). Ko so prekrvavljenost bolnikovega stegna pregledali z Dopplerskim ultrazvokom, so menili, da ima lahko bolnik arterijovenozno fistulo, ki je nastala po poškodbi. Podobnega mnenja so bili preiskovalci v pediatrični bolnici, kjer so dečka ponovno pregledali z Dopplerskim ultrazvokom. Vendar digitalna subtrakcijska angiografija, ki so jo naredili 8 mesecev po poškodbi, ni potrdila izvidov ultrazvočnih preiskav. Zato so 11 mesecev po poškodbi naredili računalniško tomografsko angiografijo, ki je jasno pokazala spremembe, ki so bile že večkrat opisane ob predhodnih ultrazvočnih preiskavah. 14 mesecev po poškodbi je bolnika operiral žilni kirurg in klinično stanje se je znatno popravilo. Kljub preiskavam in kirurškemu posegu vzrok nastanka fistule pri dečku ni popolnoma pojasnjen.

Zaključki. Ob neobičajnih ponavljajočih hematomih, ki nastanejo po poškodbi, je indicirana Dopplerska ultrazvočna priskava. Z računalniško tomografsko angiografijo laho natančneje opredelimo ultrazvočno vidne spremembe.

Radiol Oncol 2006; 40(4): 219-29.

Tumorska cepiva

Frank M, Ihan A

Tumorska cepiva imajo številne prednosti v primerjavi z drugimi oblikami zdravljenja raka. Predstavljajo visokospecifično protitumorsko terapijo in jih lahko usmerimo proti antigenom, ključnim za proces maligne preobrazbe. Imajo edinstven potencial za trajni protitumorski učinek zaradi nastanka dolgoživega, za tumor specifičnega imunskega spomina. Kljub velikim pričakovanjem so dosedanji klinični poskusi cepljenja bolnikov z rakom s tumorskimi cepivi v glavnem prinesli razočaranje. Vzroki za neuspešnost tumorskih cepiv so številni. Potencialno protitumorsko populacijo limfocitov T predstavljajo nizkoafinitetni in maloštevilni periferni limfociti T. Večina tumorskih antigenov namreč predstavlja lastne antigene, za katere je imunski sistem toleranten. Vzporedno tumorji razvijajo različne mehanizme, s katerimi se izogibajo imunskemu sistemu in so kot taki slabo imunogeni ali celo tolerogeni. Novejše imunoterapevtske strategije so usmerjene v premagovanje imunske tolerance na tumorske antigene, povečevanje imunogenosti tumorskih cepiv in nasprotovanje mehanizmom tumorskega izogibanja imunskemu sistemu. Pristopi so številni, a še vedno daleč stran od idealnega tumorskega cepiva, ki bi uspešno zavrnilo tumor. Težave pri aktivaciji protitumorskega imunskega odziva s tumorskimi cepivi so privedle do razvoja alternativnih imunoterapevtskih strategij, ki neposredno vključujejo efektorske mehanizme imunskega odziva (adoptivni prenos limfocitov T in monoklonska protitelesa).

Radiol Oncol 2006; 40(4): 231-7.

Preoperativna sočasna kemoradioterapija pri raku požiralnika

Šeruga B, Sok M, Eržen J, Jerman J, Jančar B, Zakotnik B

Izhodišča. Kirurško zdravljenje in zdravljenje s sočasno kemoradioterapijo (KRT) sta danes možna načina primarnega zdravljenja raka požiralnika. Dolgotrajno preživetje bolnikov z lokalno napredovalo boleznijo je redko. Zdravljenje teh bolnikov s preoperativno KRT bi lahko bilo koristno, zlasti pri dosegu patološkega popolnega odgovora (pPO) po KRT. V retrospektivni analizi smo analizirali učinkovitost in toksičnost preoperativne KRT pri bolnikih z lokalno napredovalim rakom požiralnika ter vpliv pPO na preživetje.

Bolniki in metode. Od leta 1996 do 2002 smo zdravili 41 bolnikov z lokalno napredovalim rakom požiralnika s cisplatinom 75 mg/m^2 in 5-FU 1000 mg/m^2 v štiridnevni kontinuirani infuziji s pričetkom 1. in 22. dan. Sočasno so prejeli 4500 cGy, 200-300 cGy/dan. Ezofagektomija je sledila 4-5 tednov po zaključeni radioterapiji. Po operaciji smo jih redno sledili na 3-6 mesecev.

Rezultati. Pri 26,8% bolnikov je bil dosežen pPO. Čas srednjega preživetja je bil 18 mesecev za vse bolnike, 21,2 meseca za bolnike s pPO in 16 mesecev za bolnike z rezidualno boleznijo (p = 0,79). Postoperativna smrtnost je bila 22%. Srednja intenziteta odmerka za cisplatin je bila 92% in za 5-FU 71,5% predvidenega celokupnega odmerka. Bolezen se je najpogosteje ponovila lokoregionalno (31,7%), celokupno se je bolezen ponovila v 43,9%.

Zaključek. Sodobnejši načini radioterapije in zadostna intenziteta odmerka bi lahko prispevali k izboljšanju lokoregionalne kontrole. Za ta kombiniran način zdravljenja je potrebna skrbna izbira bolnikov brez pridruženih sočasnih obolenj in oddaljenih zasevkov.

Radiol Oncol 2006; 40(4): 239-44.

Indeks telesne mase in tveganje za pljučnega raka pri nekadilcih

Kagohashi K, Satoh H, Kurishima K, Ishikawa H, Ohtsuka M

Izhodišča. Razmerje med indeksom telesne mase (BMI) in tveganjem za pljučnega raka pri nekadilcih do sedaj še ni bilo natančno opredeljeno. Da bi to tveganje ocenili, smo naredili študijo primerov.

Metode. V letih 1987 do 2005 smo v raziskavi obravnavali 204 bolnikov nekadilcev s pljučnim rakom in 398 bolnikov v kontrolni skupini. Starostno primerljive bolnike v kontrolni skupini smo izbrali med nekadilci, ki so se zdravili v naši bolnišnici zaradi nemaligne pljučne bolezni.

Rezultati. Ko smo primerjali starostno primerljivo skupino bolnikov z najmanjšim BMI (BMI < 20.8), nismo ugotovili manjše povezave s pljučnim rakom kot v skupinah z višjim BMI (v drugi skupini z BMI ≥ 20,8 do < 22,9, p = 0,683; v tretji skupini z BMI ≥ 22,9 do < 24,9, p = 0,745; in v skupini z najvišjim BMI, BMI ≥ 25,0, p = 0,327).

Prav tako primerjava omenjenih skupin pri ženskah ni pokazala povezave med BMI in pojavnostjo pljučnega raka (pri drugi skupini je bil p = 0,639; pri tretji p = 0,667; in pri skupini z najvišjim BMI p = 0,978).

Zaključki. Naša raziskava ni pokazala, da bi pri nekadilcih BMI bil povezan s stopnjo tveganja za pljučnega raka. Na obolevanje za pljučni rak še vedno najbolj vplivajo drugi dejavniki, največ kajenje.

Radiol Oncol 2006; 40(4): 245-57.

Pomen različnih shem zdravljenja z doksorubicinom in vinblastinom na EAT tumorjih pri miših

Auersperg M, Pogačnik A, Kloboves-Prevodnik V, Serša G, Čemažar M

Izhodišča. Določevali smo pomen različnih shem zdravljenja z doksorubicinom in vinlastinom na uspešnost zdravljenja tumorjev.
Materiali in metode. CBA miši z intraperitonealno nasajenimi Ehrlich ascitesnimi tumorji smo zdravili z doksorubicinom, vinblastinom ali kombinacijo obeh v različnih zaporedjih in časovnih intervalih.
Rezultati. Kombinacija doksorubicina in vinblastina z 48-h intervalom med aplikacijo kemoterapevtikov, ne pa tudi s 24-h, je statistično značilno zmanjšala število tumorskih celic v ascitesu v primerjavi z drugimi terapijami, ne glede na zaporedje aplikacije kemoterapevtikov. Pri kombiniranih terapijah je bil učinek na morfološke spremembe in porazdelitev DNA določen s kemoterapevtikom, ki smo ga aplicirali najprej. Ne glede na zaporedje aplikacije kemoterapevtikov se mediana časov preživetja živali ni statistično razlikovala med posameznimi skupinami.
Zaključki. Učinek kombinacije doksorubicina in vinblastina je odvisen od časovnega intervala med aplikacijo kemoterapevtikov, ne pa tudi od zaporedja. Rezultati predkliničnih študij so pomembni za načrtovanje kombiniranih kemoterapevtskih shem v klinični praksi.

Radiol Oncol 2006; 40(4): 259-71.

Cisteinski katepsini, stefini in razgradnja izvenceličnega matriksa med invazijo človeških transformiranih celičnih linij raka dojke

Zajc I, Bervar A, Lah TT

Izhodišča. Celični model človeškega raka dojke, ki je vseboval štiri celične linije, osnovno MCF10A, spontano imortalizirano linijo epitelija človeške dojke, MCF10AT, ki je nastala s c-Ha-*ras* transfekcijo osnovne linije ter dve tumorigeni liniji, MCF10AT-Ca1a in MCF10AT-Ca1d, pridobljeni iz zaporednih vsadkov MCF10AT v miškah, smo uporabili za primerjavo relativne količine katepsinov in stefinov v posameznih celicah.
Metode. Relativne količine katepsinov in stefinov v posameznih celicah smo ocenili s konfokalno mikroskopijo in z merjenjem proteinske koncentracije v celičnih homogenatih.
Rezultati. Najbolj invazivne MCF10AT celice so izražale nekajkrat več katepsina B in več stefinov, vsebovale pa so podobne koncentacije katepsina L kot osnovna, MCF10A celična linija. To je bilo povezano s petkrat višjo endocitozo substrata - Matrigela z DQ kolagenom IV (DQC) - in s hkratnim povišanjem prekrivanja signalov med DQC in katepsinom L ter DQC in stefinom B, a z manjšim prekrivanjem signalov med DQC in katepsinom B v MCF10AT celicah. Pri teh celicah smo opazili tudi znatno večje prekrivanje signalov med obema katepsinoma in med pari katepsinov in stefinov.
Zaključki. Ti rezultati kažejo, da pride pri invazivnem celičnem fenotipu do večje endocitoze in razgradnje kolagena ter istočasne spremembe v lokalizaciji katepsinov in stefinov v celici. Glede na te in podatke drugih avtorjev se zdi, da je znotrajcelična razgradnja bolj povezana s katepsinom L, medtem ko katepsin B v večji meri sodeluje pri pericelularni razgradnji medceličnega matriksa tekom celične migracije in invazije.

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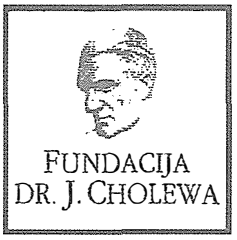

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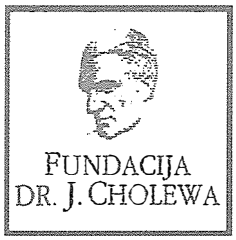

Activity of "dr. J. Cholewa" Foundation for Cancer Research and Education - a report for the final quarter of 2006

The Dr. J. Cholewa Foundation for Cancer Research and Education is preparing plans to continue to support activities associated with cancer research and education in Slovenia in the coming 2007. The Foundation remains active in promoting any form of cancer education in general population, among medical and nursing students and among all the others with a particular interest in cancer research and education. A number of requests for research grants and other forms of financial support were thus received from experts in various disciplines of cancer research and education in Slovenia in the year 2006 and needless to say, all have been dealt with great attention and responsibly in respect to their proposals and contents. The role of the Foundation members with clinical and research experience in cancer and by members with important experience in finance has been instrumental in this activity and their contribution is gratefully acknowledged.

The Dr. J. Cholewa Foundation for Cancer Research and Education also continues to support the regular publication of "Radiology and Oncology" international scientific journal, which is edited, published and printed in Ljubljana, Slovenia. In line with the philosophy of the Foundation, the spread and expansion of the information and knowledge of cancer research and problems associated with cancer in general have thus found the way to many interested professionals, lay public and others in Slovenia and elsewhere.

The support for cancer research and education in various forms, financial and otherwise, remains to be considered as one of the more important commitments of the The Dr. J. Cholewa Foundation. It is important to note that a number research and study grants have been bestowed and allocated by the Foundation to researchers from various scientific spheres of acticvity associated with oncology in Slovenia in the year 2006. Other forms of support were also allotted to attend scientific meetings, conferences and symposia dealing with oncology worldwide to a number of interested and qualified applicants. The Foundation will therefore also support the publication of the results from cancer research in Slovenia in any respectable international scientific journal and in any other form of dissemination of scientific information.

The Dr. J. Cholewa Foundation for Cancer Research and Education has since its inception every reason to respectfully acknowledge the importance of various forms of support from various public companies and private individuals to its cause. With best wishes for the year 2007, the Foundation extends its thanks and gratitude to all of them.

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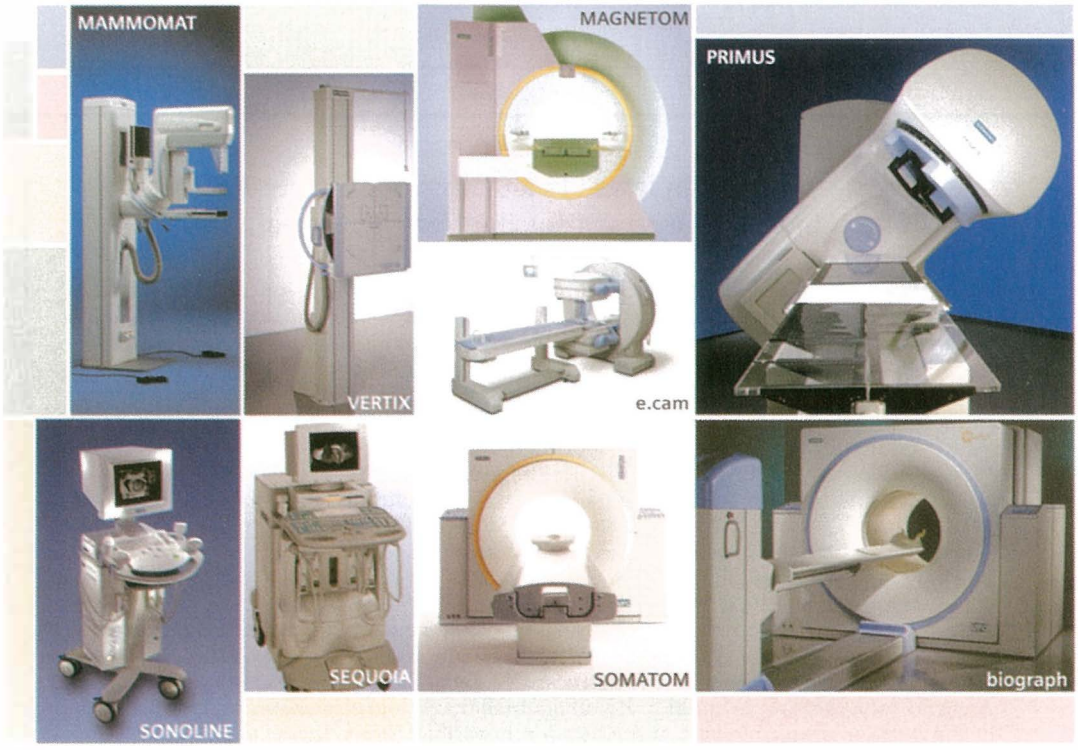

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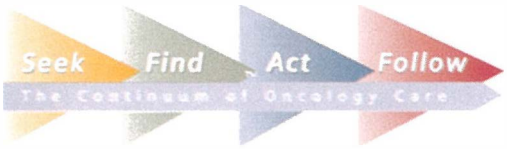
Every day in the United States alone, 29,000 cancer patients receive radiation therapy delivered by Siemens linear accelerators. As clinical protocols transition to include IMRT and IGRT, Siemens seamlessly integrates the diagnostic and treatment modalities. That's what we call **Best Practice Oncology Care**.



Siemens medical
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ZASTOPA PODJETJA:

MENTOR

Prsni vsadki napolnjeni s silikonskim gelom, ekspanderji in drugi pripomočki pri rekonstrukciji dojk

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

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

Angelantoni scientifica (Italija):
hladilna tehnika in aparati za laboratorije, transfuzijo, patologijo in sodno medicino

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

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

Sakura finetek (Evropa):
aparati za pripravo histoloških preparatov: mikro-inkriotomi, zalivalci, tkivni procesorji, barvalci, pokrivalci

Micronic (Nizozemska):
sistemi za shranjevanje vzorcev, pipete, nastavki za pipete

Integra Biosciens (Švica):
laboratorijska oprema za mikrobiologijo, biologijo celic, molekularno biologijo in biotehnologijo

Implantech (Amerika):
obrazni in glutealni vsadki

SpectrumDesigns MEDICAL (Amerika):
moški pektoralni vsadki

Biomerica (Amerika):
hitri testi za diagnostiko, EIA /RIA testi

Byron (Amerika):
liposuktorji in kanile za liposukcijo

LABORMED d.o.o.
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Erbitux 2 mg/ml raztopina za infundiranje (skrajšana navodila za uporabo)
Cetuksimab je monoklonsko IgG1 protitelo, usmerjeno proti receptorju za epidermalni rastni faktor (EGFR). Terapevtske indikacije: Zdravilo Erbitux je v kombinirani terapiji z irinotekanom indicirano za zdravljenje bolnikov z metastatskim rakom debelega črevesa in danke, in sicer po neuspešni citotoksični terapiji, ki je vključevala tudi irinotekan. Zdravilo Erbitux je v kombinaciji z radioterapijo indicirano za zdravljenje bolnikov z lokalno napredovalim rakom skvamoznih celic glave in vratu. Odmerjanje in način uporabe: Zdravilo Erbitux 2 mg/ml se daje z intravensko infuzijo prek linijskega filtra. Zdravilo Erbitux pri vseh indikacijah infundirajte enkrat na teden. Začetni odmerek je 400 mg cetuksimaba/m² telesne površine, vsi naslednji tedenski odmerki so vsak po 250 mg/m². Pred prvo infuzijo mora bolnik prejeti premedikacijo z antihistaminikom. Ta premedikacija je priporočljiva tudi pred vsemi naslednjimi infuzijami. Kontraindikacije: Zdravilo Erbitux je kontraindicirano pri bolnikih z znano hudo preobčutljivostno reakcijo (3. ali 4. stopnje) na cetuksimab. Pred začetkom kombiniranega zdravljenja morate upoštevati kontraindikacije za irinotekan ali radioterapijo. Posebna opozorila in previdnostni ukrepi: če pri bolniku nastopi blaga ali zmerna reakcija, povezana z infundiranjem, lahko zmanjšate hitrost infundiranja. Priporočljivo je, da ostane hitrost infundiranja na nižji vrednosti tudi pri vseh naslednjih infuzijah. Če se pri bolniku pojavi huda kožna reakcija (≥ 3. stopnje po kriterijih NCI-CTC), morate prekiniti terapijo s cetuksimabom. Z zdravljenjem smete nadaljevati le, če se je reakcija pomirila do 2. stopnje. Posebna previdnost je potrebna pri oslabljenih bolnikih in pri tistih z obstoječo srčno-pljučno boleznijo. Neželeni učinki: Zelo pogosti (≥ 1/10): dispneja, blago do zmerno povečanje ravni jetrnih encimov, kožne reakcije, blage ali zmerne reakcije, povezane z infundiranjem, blag do zmeren mukozitis. Pogosti (≥ 1/100, < 1/10): konjunktivitis, hude reakcije, povezane z infundiranjem. Pogostost ni znana: hipomagneziemija. Pakiranje: 1 viala po 50 ml. Imetnik dovoljenja za promet: Merck KGaA, 64271 Darmstadt, Nemčija. Podrobne informacije o zdravilu so objavljene na spletni strani Evropske agencije za zdravila (EMEA) http://www.emea.eu.int/

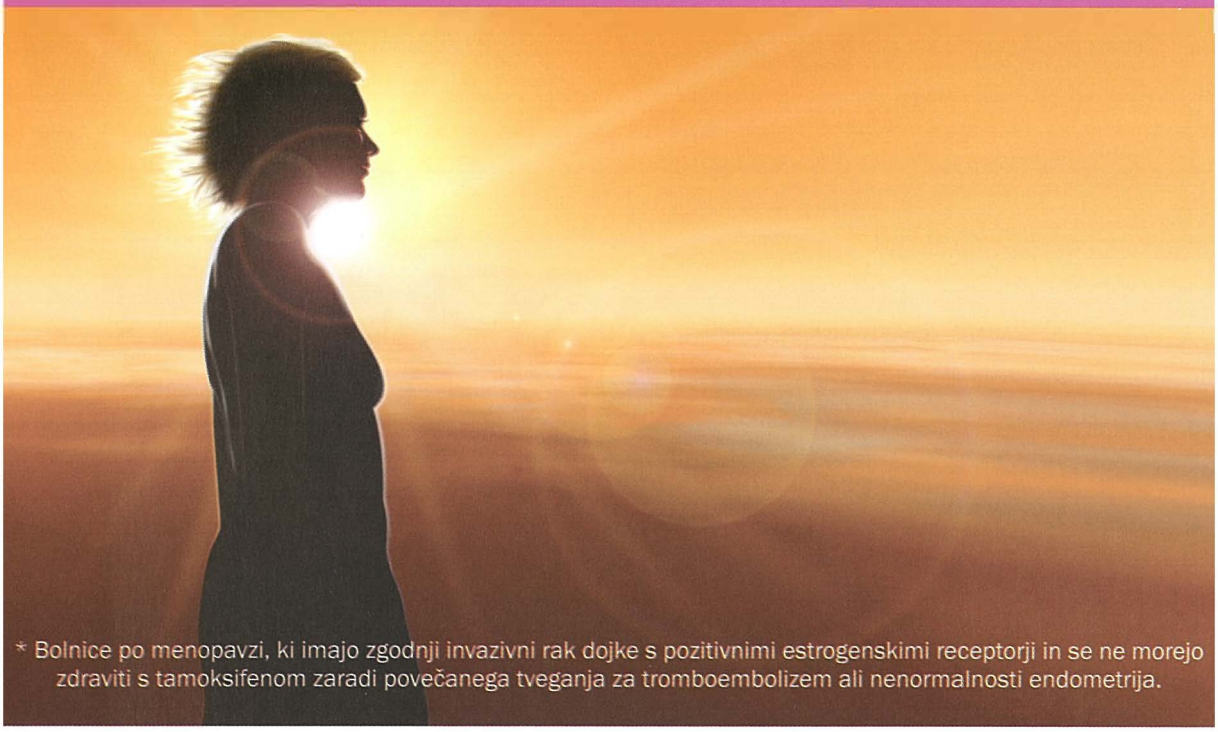
Dodatne informacije so vam na voljo pri: Merck, d.o.o., Dunajska cesta 119, 1000 Ljubljana, tel.: 01 560 3810, faks: 01 560 3831, el. pošta: info@merck.si

[1] ploščatocelični rak glave in vratu
[2] v primerjavi z radioterapijo
[3] Bonner et al. Radiotherapy plus Cetuximab for Squamous - Cell Carcinoma of the Head and Neck. N Engl J Med 2006; 354(6): 567-78

www.oncology.merck.de

MERCK

dopolnilno zdravljenje*



* Bolnice po menopavzi, ki imajo zgodnji invazivni rak dojke s pozitivnimi estrogenskimi receptorji in se ne morejo zdraviti s tamoksifenom zaradi povečanega tveganja za tromboembolizem ali nenormalnosti endometrija.

Arimidex vodilni zaviralec aromataze

anastrozol

Kratka informacija o zdravilu Arimidex 1 mg

estava: Filmsko obložena tableta vsebuje mg anastrozola.

dikacije: Adjuvantno zdravljenje žensk po enopavzi, ki imajo zgodnji invazivni rak ojke s pozitivnimi estrogenskimi receptorji in e ne morejo zdraviti s tamoksifenom zaradi ovečanega tveganja za tromboembolizem ali enormalnosti endometrija. Zdravljenje apredovalega raka dojke pri ženskah po enopavzi. Učinkovitost pri bolnicah z egativnimi estrogenskimi receptorji ni bila okazana razen pri tistih, ki so imele edhodno pozitiven klinični odgovor na moksifen.

dmerjanje in način uporabe: 1 tableta po 1 g peroralno, enkrat na dan. Pri zgodnjem ku je priporočljivo trajanje zdravljenja 5 let.

ontraindikacije: Arimidex je kontraindiciran i: ženskah pred menopavzo, nosečnicah in oječih materah, bolnicah s hujšo ledvično lpovedjo (očistek kreatinina manj kot 20 l/min (oziroma 0,33 ml/s)), bolnicah z nemim do hudim jetrnim obolenjem in olnicah, ki imajo znano preobčutljivost za nastrozol ali za katerokoli drugo sestavino lravila. Zdravila, ki vsebujejo estrogen, ne nete dajati sočasno z Arimidexom, ker bi se egovo farmakološko delovanje izničilo. moksifena se ne sme uporabljati skupaj z imidexom, ker lahko pride do zmanjšanja njegovega delovanja.

Posebna opozorila in previdnostni ukrepi: Uporabe Arimidexa ne priporočamo pri otrocih, ker njegova varnost in učinkovitost pri njih še nista raziskani. Menopavzo je potrebno biokemično določiti pri vseh bolnicah, kjer obstaja dvom o hormonskem statusu. Ni podatkov o varni uporabi Arimidexa pri bolnicah z zmerno ali hudo jetrno okvaro ali hujšo ledvično odpovedjo (očistek kreatinina manj kakor 20 ml/min (oziroma 0,33 ml/s)). Ni podatkov o uporabi anastrozola z analogi LHRH. Te kombinacije zdravil se ne sme uporabljati zunaj kliničnih preskušanj. Pri ženskah z osteoporozo ali pri ženskah s povečanim tveganjem za razvoj osteoporoze je treba določiti njihovo mineralno gostoto kosti z denzitometrijo, na primer s slikanjem DEXA na začetku zdravljenja, pozneje pa v rednih intervalih. Po potrebi je treba začeti z zdravljenjem ali preprečevanjem osteoporoze in to skrbno nadzorovati. Ni verjetno, da bi Arimidex zmanjšal bolničino sposobnost za vožnjo ali upravljanje s stroji. Ker pa so med uporabo Arimidexa poročali o splošni oslabelosti in zaspanosti, je potrebna previdnost pri vožnji in upravljanju strojev, dokler simptoma trajata.

Nosečnost in dojenje: Arimidex je med nosečnostjo in dojenjem kontraindiciran.

Neželeni učinki: Najpogostejši neželeni učinki so navali vročine, suhost vagine in redčenje las. Ostali neželeni učinki vključujejo gastrointestinalne motnje (anoreksija, slabost, bruhanje, diareja), astenijo, bolečine/okorelost v sklepih, zaspanost, glavobol in izpuščaje. Občasna poročila navajajo krvavitev iz nožnice, ki se pretežno pojavlja pri bolnicah z napredovalim obolenjem raka na dojki v prvih tednih po prehodu z dotedanjega hormonskega zdravljenja na zdravljenje z Arimidexom. Če krvavitev traja dlje časa, so potrebne dodatne preiskave. Hiperholesterolemija, običajno blaga do zmerna. O povišanih nivojih gama-GT in alkalne fosfataze so poročali le občasno. Vzročna povezanost omenjenih sprememb ni bila ugotovljena.

Medsebojno delovanje z drugimi zdravili: Zdravila, ki vsebujejo estrogen, ne smete dajati sočasno z Arimidexom, ker bi se njegovo farmakološko delovanje izničilo. Tamoksifena se ne sme uporabljati skupaj z Arimidexom, ker lahko pride do zmanjšanja njegovega delovanja.

Vrsta ovojnine in vsebina: Pretisni omoti iz PVC in aluminija, ki vsebujejo 28 tablet v škatlici.

Režim izdaje zdravila: Rp/Spec

Datum priprave informacije: september 2006

Pred predpisovanjem, prosimo, preberite celoten povzetek temeljnih značilnosti zdravila.

odatne informacije in literatura so na voljo pri:
straZeneca UK Limited, Podružnica v Sloveniji, Verovškova 55, Ljubljana
ww.breastcancersource.com
ww.arimidex.net

AstraZeneca
ONKOLOGIJA

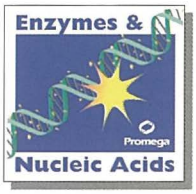
KEMOMED

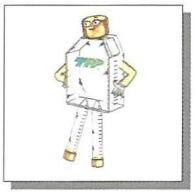
PE: Stritarjeva 5, 4000 Kranj, Slovenija
tel.: (0)4/ 2015 050, fax: (0)4/ 2015 055
e-mail: kemomed@siol.net,
www.kemomed.si

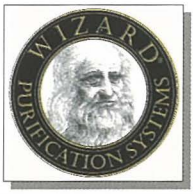
Promega

SYNGENE

TRP







IZDELKI ZA MOLEKULARNO BIOLOGIJO

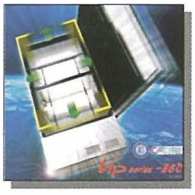
DOKUMENTACIJA IN ANALIZA GELOV

PLASTIKA ZA CELIČNE KULTURE

ELGA LABWATER

SANYO

Invitrogen life technologies



ČISTA VODA ZA LABORATORIJ

SKRINJE IN HLADILNIKI

CELIČNE KULTURE, GELI IN MOLEKULARNA BIOLOGIJA

BIOHIT

mi er va biolabs

LI-COR Biosciences

ELEKTRONSKE IN MEHANSKE AVTOMATSKE PIPETE

DIAGNOSTIKA MIKOPLAZEM IN LEGIONEL

SEKVENATORJI

Temodal 20 mg; 100 mg, 250 mg. Sestava zdravila Vsaka kapsula zdravila Temodal vsebuje 20 mg, 100 mg ali 250 mg temozolamida.**Terapevtske indikacije** Temodal kapsule so indicirane za zdravljenje bolnikov z: - za zdravljenje novo diagnosticiranega glioblastoma multiforme, sočasno z radioterapijo in kasneje kot monoterapija, - malignim gliomom, na primer multiformnim glioblastomom ali anaplastičnim astrocitomom, ki se po standardnem zdravljenju ponovi ali napreduje. **Odmerjanje in način uporabe** Temodal smejo predpisati le zdravniki, ki imajo izkušnje z zdravljenjem možganskih tumorjev. **Odrasli bolniki z novo diagnosticiranim glioblastomom multiforme** Temodal se uporablja v kombinaciji z žariščno radioterapijo (faza sočasne terapije), temu pa sledi do 6 ciklov monoterapije z temozolomidom.**Faza sočasne terapije** Zdravilo Temodal naj bolnik jemlje peroralno v odmerku 75 mg/m² na dan 42 dni, sočasno z žariščno radioterapijo (60 Gy, danih v 30 delnih odmerkih). Odmerka ne boste zmanjševali, vendar se boste vsak teden odločili o morebitni odložitvi jemanja temozolomida ali njegovi ukinitvi na podlagi kriterijev hematološke in nehematološke toksičnosti. Zdravilo Temodal lahko bolnik jemlje ves čas 42-dnevnega obdobja sočasne terapije do 49 dni, če so izpolnjeni vsi od naslednjih pogojev: absolutno število nevtrofilcev $\geq$ 1,5 x 10^9/l, število trombocitov $\geq$ 100 x 10^9/l, skupni kriteriji toksičnosti (SKT) za nehematološko toksičnost $\leq$ 1. stopnje (z izjemo alopecije, slabosti in bruhanja). Med zdravljenjem morate pri bolniku enkrat na teden pregledati celotno krvno sliko. **Faza monoterapije** Štiri tedne po zaključku faze sočasnega zdravljenja z zdravilom Temodal in radioterapijo naj bolnik jemlje zdravilo Temodal do 6 ciklov monoterapije. V 1. ciklu (monoterapija) je odmerek zdravila 150 mg/m² enkrat na dan 5 dni, temu pa naj sledi 23 dni brez terapije. Na začetku 2. cikla odmerek povečajte na 200 mg/m², če je SKT za nehematološko toksičnost za 1. cikel stopnje $\leq$ 2 (z izjemo alopecije, slabosti in bruhanja), absolutno število nevtrofilcev (ASN) $\geq$ 1,5 x 10^9/l in število trombocitov $\geq$ 100 x 10^9/l. Če odmerka niste povečali v 2. ciklusu, ga v naslednjih ciklusih ne smete povečevati. Ko pa odmerek enkrat povečate, naj ostane na ravni 200 mg/m² na dan v prvih 5 dneh vsakega naslednjega ciklusa, razen če nastopi toksičnost. Med zdravljenjem morate pregledati celotno krvno sliko na 22. dan (21 dni po prvem odmerku zdravila Temodal). **Ponavljajoči ali napredujoči maligni gliom: Odrasli bolniki** Posamezen ciklus zdravljenja traja 28 dni. Bolniki, ki še niso bili zdravljeni s kemoterapijo, naj jemljejo Temodal peroralno v odmerku 200 mg/m² enkrat na dan prvih 5 dni, temu pa naj sledi 23-dnevni premor (skupaj 28 dni). Pri bolnikih, ki so že bili zdravljeni s kemoterapijo, je začetni odmerek 150 mg/m² enkrat na dan, v drugem ciklusu pa se poveča na 200 mg/m² enkrat na dan 5 dni, če ni bilo hematoloških toksičnih učinkov. **Pediatrični bolniki** Pri bolnikih, starih 3 leta ali starejših, posamezen ciklus zdravljenja traja 28 dni. Temodal naj jemljejo peroralno v odmerku 200 mg/m² enkrat na dan prvih 5 dni, potem pa naj sledi 23-dnevni premor (skupaj 28 dni). Otroci, ki so že bili zdravljeni s kemoterapijo, naj prejmejo začetni odmerek 150 mg/m² enkrat na dan 5 dni, s povečanjem na 200 mg/m² enkrat na dan 5 dni v naslednjem ciklusu, če ni bilo hematoloških toksičnih učinkov. **Bolniki z motnjami v delovanju jeter ali ledvic** Pri bolnikih z blagimi ali zmernimi motnjami v delovanju jeter je farmakokinetika temozolomida podobna kot pri tistih z normalnim delovanjem jeter. Podatki o uporabi zdravila Temodal pri bolnikih s hudimi motnjami v delovanju jeter (razred III po Child-u) ali motnjami v delovanju ledvic niso na voljo. Na podlagi farmakokinetičnih lastnosti temozolomida obstaja majhna verjetnost, da bo pri bolnikih s hudimi motnjami v delovanju jeter ali ledvic potrebno zmanjšanje odmerka zdravila. Kljub temu je potrebna previdnost pri uporabi zdravila Temodal pri teh bolnikih. **Starejši bolniki** Analiza farmakokinetike je pokazala, da starost ne vpliva na očistek temozolomida. Kljub temu je potrebna posebna previdnost pri uporabi zdravila Temodal pri starejših bolnikih. **Način uporabe** Temodal mora bolnik jemati na tešče. Temodal kapsule mora bolnik pogoltniti cele s kozarcem vode in jih ne sme odpirati ali žvečiti. Predpisani odmerek mora vzeti v obliki najmanjšega možnega števila kapsul. Pred jemanjem zdravila Temodal ali po njem lahko bolnik vzame antiemetik. Če po zaužitju odmerka bruha, ne sme še isti dan vzeti drugega odmerka. **Kontraindikacije** Temodal je kontraindiciran pri bolnikih, ki imajo v anamnezi preobčutljivostne reakcije na sestavine zdravila ali na dakarbazin (DTIC). Temodal je kontraindiciran tudi pri bolnikih s hudo mielosupresijo. Temodal je kontraindiciran pri ženskah, ki so noseče ali dojijo. **Posebna opozorila in previdnostni ukrepi** Pilotno preskušanje podaljšane 42-dnevne sheme zdravljenja je pokazalo, da imajo bolniki, ki so sočasno prejemali zdravilo Temodal in radioterapijo, še posebej veliko tveganje za nastanek pljučnice zaradi okužbe s *Pneumocystis carinii* (PCP). Profilaksa proti tovrstni pljučnici je torej potrebna pri vseh bolnikih, ki sočasno prejemajo zdravilo Temodal in radioterapijo v okviru 42-dnevne sheme zdravljenja (do največ 49 dni), ne glede na število limfocitov. Če nastopi limfopenija, mora bolnik nadaljevati s profilakso, dokler se limfopenija ne povrne na stopnjo $\leq$ 1. **Antiemetična terapija:** Z jemanjem zdravila Temodal sta zelo pogosto povezana slabost in bruhanje. **Laboratorijske vrednosti** Pred jemanjem zdravila morata biti izpolnjena naslednja pogoja za laboratorijske izvide: ANC mora biti $\geq$ 1,5 x 10^9/l in število trombocitov $\geq$ 100 x 10^9/l. Na 22. dan (21 dni po prvem odmerku) ali v roku 48 ur od navedenega dne, morate pregledati celotno krvno sliko in jo nato spremljati vsak teden, dokler ni ANC nad 1,5 x 10^9/l in število trombocitov nad 100 x 10^9/l. Če med katerimkoli ciklusom ANC pade na < 1,0 x 10^9/l ali število trombocitov na < 50 x 10^9/l, morate odmerek zdravila v naslednjem ciklusu zmanjšati za eno odmerno stopnjo. Odmerne stopnje so 100 mg/m², 150 mg/m² in 200 mg/m². Najmanjši priporočeni odmerek je 100 mg/m². **Moški bolniki** Temozolomid lahko deluje genotoksično, zato morate moškim, ki se zdravijo z temozolomidom svetovati, da naj ne zaplodijo otroka še šest mesecev po zdravljenju. **Interakcije** Sočasna uporaba zdravila Temodal in ranitidina ni povzročila spremembe obsega absorpcije temozolomida ali monometiltriazenoimidazol karboksamida (MTIC). Jemanje zdravila Temodal s hrano je povzročilo 33 % zmanjšanje Cmax in 9 % zmanjšanje površino pod krivuljo (AUC). Ker ne moremo izključiti možnosti, da bi bila sprememba Cmax lahko klinično pomembna, naj bolniki jemljejo zdravilo Temodal brez hrane. Analiza populacijske farmakokinetike v preskušanjih druge faze je pokazala, da sočasna uporaba deksametazona, proklorperazina, fenitoina, karbamazepina, ondansetrona, antagonistov receptorjev H2 ali fenobarbitala ne spremeni očistka temozolomida. Sočasno jemanje z valprojsko kislino je bilo povezano z majhnim, a statistično značilnim zmanjšanjem očistka temozolomida. Uporaba zdravila Temodal v kombinaciji z drugimi mielosupresivnimi učinkovinami lahko poveča verjetnost mielosupresije. **Nosečnost** Študij na nosečih ženskah ni bilo. Predklinične študije na podganah in kuncih z odmerkom 150 mg/m2 so pokazale teratogenost in/ali toksičnost za plod. Zato naj noseče ženske načeloma ne bi jemale zdravila Temodal. Če pa je uporaba v času nosečnosti nujna, morate bolnico opozoriti na možne nevarnosti zdravila za plod. Ženskam v rodni dobi svetujte, naj med zdravljenjem z zdravilom Temodal preprečijo zanositev. **Dojenje** Ni znano, ali se temozolomid izloča v materino mleko, zato ženske, ki dojijo, ne smejo jemati zdravila Temodal. **Neželeni učinki** V kliničnih preskušanjih so bili najpogostnejši neželeni učinki, povezani z zdravljenjem, prebavne motnje, natančneje slabost (43 %) in bruhanje (36 %). Oba učinka sta bila ponavadi 1. ali 2. stopnje (od 0 do 5 epizod bruhanja v 24 urah) in sta prenehala sama ali pa ju je bilo mogoče hitro obvladati s standardnim antiemetičnim zdravljenjem. Incidenca hude slabosti in bruhanja je bila 4 %. **Laboratorijski izvidi:** Trombocitopenija in nevtropenija 3. in. 4. stopnje sta se pojavili pri 19 % in 17 % bolnikov, zdravljenih zaradi malignega gliома. Zaradi njiju je bila potrebna hospitalizacija in/ali prekinitev zdravljenja z zdravilom Temodal pri 8 % in. 4 % bolnikov. Mielosupresija je bila predvidljiva (ponavadi je je pojavila v prvih nekaj ciklusih in je bila najizrazitejša med 21. in 28. dnem), okrevanje pa je bilo hitro, ponavadi v 1 do 2 tednih. Opazili niso nobenih dokazov kumulativne mielosupresije. Trombocitopenija lahko poveča tveganje za pojav krvavitev, nevtropenija ali levkopenija pa tveganje za okužbe.**Imetnik dovoljenja za promet** SP Europe 73, rue de Stalle B-1180, Bruselj, Belgija. **Način in režim izdaje** Zdravilo se izdaja samo na recept, uporablja pa se pod posebnim nadzorom zdravnika specialista ali od njega pooblaščenega zdravnika. **Datum priprave informacije** januar 2006 Podrobnejše informacije o zdravilu Temodal dobite na sedežu podjetja.

Priprava informacije Schering-Ploug, november 2006.

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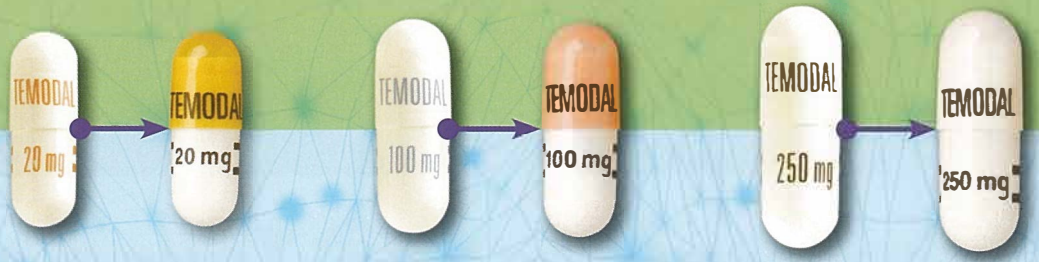

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1- Tarceva (erlotinib) summary of product characteristics, F.Hoffmann-La Roche LTD., 2005.

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UKRC 2007 - Advances in Technology

UKRC 2007 should prove to be one of the most stimulating congresses to date in terms of the scientific coverage of new and developing fields within medical imaging. Key sessions will build on the latest technology being presented within the technical exhibition, providing a unique multi-disciplinary forum to exchange ideas about the latest developments in technology which will impact on radiology and clinical applications.

Monday sees a revisit to a very popular session from 2006 on image perception, featuring an international cast from Europe and the USA, a key update on radiation protection and x-ray equipment performance issues, including Dr. Walter Huda from the USA presenting a new paradigm for CT dosimetry. This first day also involves a look at new emerging imaging techniques such as PET/MR systems and what promises to be a very poignant debate, hosted by Prof. Mike Smith and Dr. Giles Maskell, on whether advances in technology will render the radiologists and radiographer redundant.

Tuesday morning has a session on the latest advances in cartilage imaging and two sessions on developments in MR imaging, incorporating presentations on blood pool agents by Dr. Giles Roditi, molecular MRI by Dr. Arne Hengerer from Erlangen and MR elasticity imaging by Dr. Ralph Sinkus from Paris. The afternoon covers quantitative imaging applications in medicine and a key session on CT with Prof. Mathias Prokop from Utrecht addressing the future role of multi slice CT, dual source CT described by Prof. Thomas Flohr from Erlangen, and 256 slice CT systems from Toshiba.

The pace fails to slow down for the final day on Wednesday, so it's best not to have too late a night at Tiger-Tiger on the Tuesday night! The scientific sessions involve a look at interactive imaging applications for surgery, including interventional MR, stereotaxy in neurosurgery and surgical robotics along with a constantly topical look at the changing face of cardiac CT. The afternoon sees a CT teaching course for radiographers, clinicians, and scientists with everything you need to know about physics, technology, image quality and patient dose.

The advances in technology sessions provide a unique opportunity to view and appreciate developing areas of radiology from a truly multi-disciplinary standpoint. Where else could you meet colleagues of all disciplines and enjoy stimulating presentations from clinicians, scientists and radiographers; not to mention surgeons and cardiologists? Be there in Manchester or be disappointed!

Best wishes,
Mr Andrew Jones
Vice President, UKRC Advances in Technology
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General instructions • Radiology and Oncology will consider manuscripts prepared according to the Vancouver Agreement (*N Engl J Med* 1991; **324:** 424-8, *BMJ* 1991; **302:** 6772; *JAMA* 1997; **277:** 927-34.). Type the manuscript double spaced on one side with a 4 cm margin at the top and left hand side of the sheet. Write the paper in grammatically and stylistically correct language. Avoid abbreviations unless previously explained. The technical data should conform to the SI system. The manuscript, including the references may not exceed 15 typewritten pages, and the number of figures and tables is limited to 4. If appropriate, organize the text so that it includes: Introduction, Material and methods, Results and Discussion. Exceptionally, the results and discussion can be combined in a single section. Start each section on a new page, and number each page consecutively with Arabic numerals.

Title page should include a concise and informative title, followed by the full name(s) of the author(s); the institutional affiliation of each author; the name and address of the corresponding author (including telephone, fax and e-mail), and an abbreviated title. This should be followed by the *abstract page*, summarising in less than 200 words the reasons for the study, experimental approach, the major findings (with specific data if possible),

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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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Results should be presented clearly and concisely without repeating the data in the tables and figures. Emphasis should be on clear and precise presentation of results and their significance in relation to the aim of the investigation.

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Illustrations and tables must be numbered and referred to in the text, with appropriate location indicated in the text margin. Illustrations must be labelled on the back with the author's name, figure number and orientation, and should be accompanied by a descriptive legend on a separate page. Line drawings should be supplied in a form suitable for high-quality reproduction. Photographs should be glossy prints of high quality with as much contrast as the subject allows. They should be cropped as close as possible to the area of interest. In photographs mask the identities of the patients. Tables should be typed double spaced, with descriptive title and, if appropriate, units of numerical measurements included in column heading.

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Dent RAG, Cole P. *In vitro maturation of monocytes in squamous carcinoma of the lung. Br J Cancer* 1981; 43: 486-95.

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Evans R, Alexander P. Mechanisms of extracellular killing of nucleated mammalian cells by macrophages. In: Nelson DS, editor. *Immunobiology of macrophage.* New York: Academic Press; 1976. p. 45-74.

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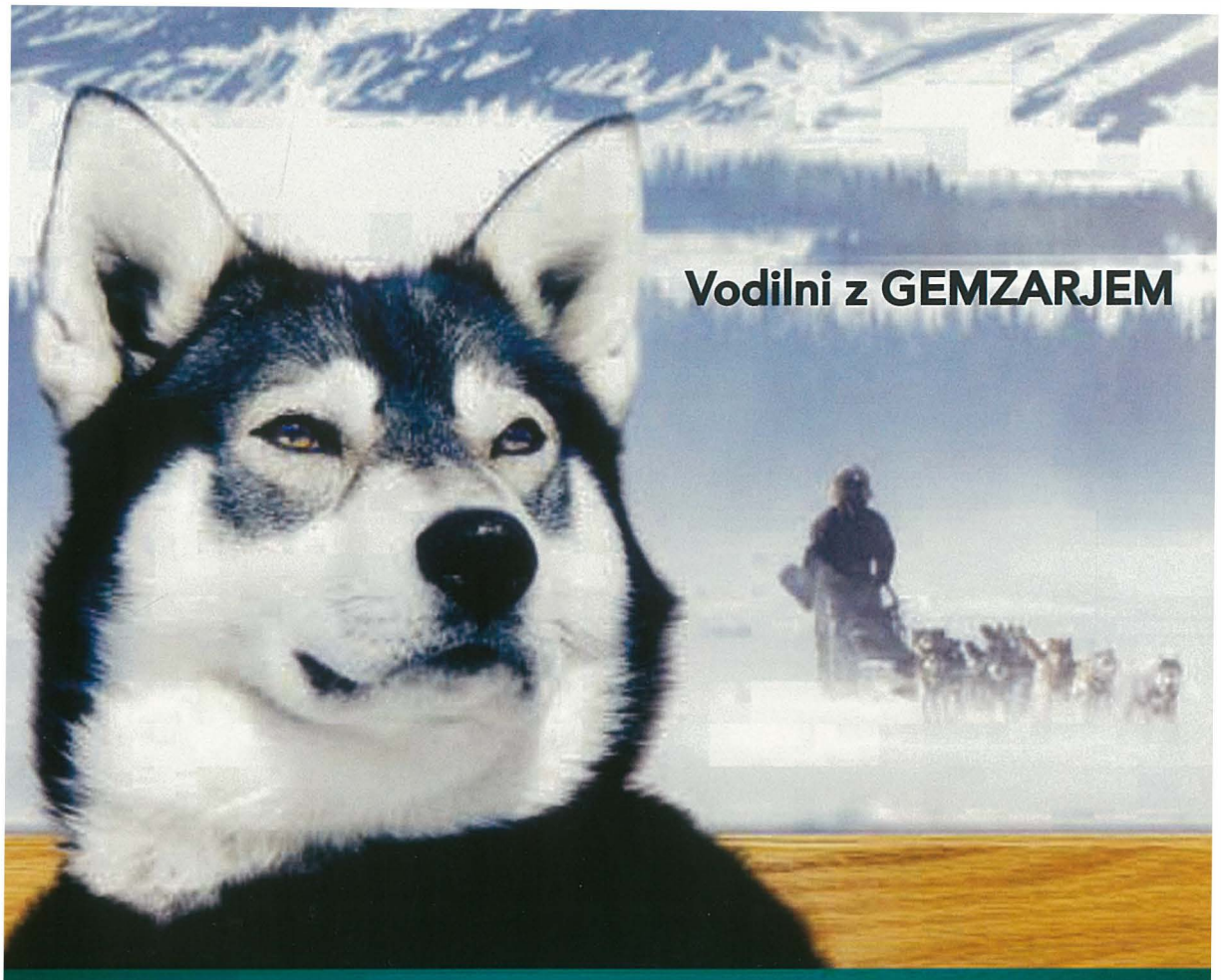

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