INSTITUTE OF ONCOLOGY, LJUBLJANA, YUGOSLAVIA

SOLUBLE TUMOR PRODUCTS INHIBIT NORMAL MONONUCLEAR CELLS PROPERTIES AND ENHANCE TUMOR GROWTH*

Serša G., Krošl G., Batista U., Rudolf Z.

Abstract — Conditioned media (CM) containing soluble tumor products were prepared from confluent cell cultures, growing in serum free media. In vitro closely related mononuclear cells (MNC) properties (adherence, migration and spreading) were influenced by CM from three different murine tumor cell lines. Autologous sarcoma (Sa 1) CM and also heterologous melanoma B 16 and Lewis lung ca (LLC) CM influenced the normal MNC properties from A/J mice. Minimal effect was observed with B 16 melanoma CM compared to Sa 1 and LLC CM. In vivo, tumor products applied systemically, either intravenously or intraperitoneally, enhanced autologous tumor growth rate. The nonspecifity of tumor factors on tumor-growth-promoting properties was observed also across the strain barrier. Growth promoting effects was observed with LLC CM on subcutaneous Sa 1 tumor bearing mice, after intravenous injection of CM. On the contrary, the LLC CM applied intraperitoneally continuosly every second day, in intramuscular B 16 melanoma bearing mice, was minimal.

UDC: 616-006.6-092.19:616.155.33-007.41

Key words: cell line, tumor products, monocytes, tumor growth, mice

Orig. sc. paper

Radiol. Iugosl. 20(3) 293—299, 1986

Introduction — Suppression of host immune functions occurs concomitantly with tumor growth, however the mechanisms mediating the tumor associated immunosuppression remain unknown. It is well established that mononuclear cells (MNC) play an important role in host defense mechanisms alone or in cooperation with specific lymphocyte-mediated mechanisms (7, 12). Various murine and human tumor-cell-derived factors have been described that suppress immune MNC functions as assessed by variety of technics (3, 4, 9, 10). The influence of tumor factors has been determined by impaired chemotactic responsiveness, enhanced migration, inhibition of phagocytosis, spreading, macrophage mediated cytotoxicity and inhibition of normal cell proliferation (1, 2, 6, 8, 13, 14, 15).

On the basis of these experiments it was postulated that the escape of tumor cells from immunotherapeutic effector mechanisms and survival of disseminated cells may be partly due to direct and indirect effects of these tumor derived immunosuppressive factors (12, 16). In the experiments investigating the enhanced tumor outgrowth, associated with tumor blocking factors, specific and nonspecific components in tumor fluids were determined. The enhancement was best demonstrated when tumor fluids were inoculated together with tumor cells, while less pronounced enhancement was observed at systemical application (5).

Therefore, we tried to compare the influence of various conditioned media (CM) derived from tumor cell cultures on in vitro MNC function, and the influence of systemic CM application on subcutaneous and intramuscular tumor growth rate in vivo. We tried to compare the influence of CM from different tumor cell lines no in vitro properties of normal MNC and on tumor growth rate, especially concerning the influence of heterologous CM on tumor growth with regard to existence of nonspecific factors in CM. As demonstrated, blocking of in vitro MNC properties and enhancement of tumor growth were ob-

* This work was supported by the Research Community of Slovenia, Grant No. C3-0563-302-86.

Received: May 5, 1986 — Accepted: June 10, 1986

served with either autologous or heterologous CM.

Material and methods — Cell lines: In the experiments nonmalignant diploid lung fibroblasts of Chinese hamster (V-79 379 A), Baby hamster kidney fibroblasts (BHK) and primary culture of A/J mice kidney fibroblasts were used. The malignant cell lines were melanoma B 16, sarcoma (Sa 1) and Lewis lung carcinoma (LLC).

Conditioned media preparation: Cell cultures were grown in Eagle minimal assential medium (EMEM) supplemented with 10 % fetal calf serum (FCS), penicillin (100 units per ml) and streptomycin (100 μg/ml) at 37° C in CO_2 incubator. Confluent cultures were washed and further grown in serum free media. After 24^h the cell culture media were collected, centrifuged 30 min at 3000 g and stored at —30° C. Protein concentration in conditioned media (CM) was adjusted in the range 500—700 μg/ml, calcium concentration was in the range 3.2—4.4 m mole/l.

Assay of peritoneal MNC adherence inhibition: MNC were obtained by washing the peritoneal cavity of healthy A/J mice with EMEM. Pooled cells from several mice were washed twice in serum free medium and their concentration adjused. 1×10^6 MNC cells and different volumes of CM were mixed in 1 ml volume. The cell suspension was thereafter incubated in hemocytometer for two hours, atfer that coverslips were gently floated away in phosphate buffer and the adhered cells rinsed several times. The adhered cells were counted and the percentage of adherence calculated. Also the MNC adherence inhibition was determined as follows:

$$\% \text{ MAI} = 100 - \frac{\% \text{ adhered cells in CM}}{\% \text{ adhered cells in EMEM}} \times 100$$

Assay of MNC spreading: Peritoneal exudate cells were collected from A/J mice and washed twice in EMEM. 2×10^6 MNC were seeded in Petri dishes (30 mm in triplicate and 50 % of different CM added to the cell cultures. After 24 hours nonadherent cells were washed away, the remaining adhered cells fixed with methanol and stained with May-Grünwald Giemsa. The spread cells with elongated processes and rounded cells with minimal to non elongated processes were scored.

Assay of MNC migration: The migration assay was performed by peritoneal exudate cells from A/J mice. Approximately 1×10^6 MNC were drawn into sterile glass capillary tube (15 × 100 mm) and sealed at one end with paraffin and vaseline. The capillaries were centrifugated (200 g for 6 min) and cut at the cell fluid interface. Three tubes were placed in each well filled with CM, covered with sterile coverslips and plates incubated for 24^h at 37° C in humid atmosphere. The migration area was projected onto the paper and measured. Results were expressed as migration index (i %).

$$i\,\% = \frac{100 \times \text{MNC migration in CM}}{\text{MNC migration in EMEM}} - 100$$

Tumor transplantation and determination of tumor volumes: Tumor cell suspensions were prepared from viable tumor pieces by mechanical disaggregation through the steel mesh. Tumors were im planted subcutaneously (Sc) or intramuscularly (Im) with 2×10^6 viable cells determined by Trypan Blue exclusion. Sc growing tumors were measured by determining 3 perpendicular tumor diameters with caliper. Im tumors were measured in the same way as Sc tumors, substracting the thickness of the contralateral leg. Tumor volumes were calculated from the formula for rotating ellipsoid.:

$$V = \frac{\pi}{6} \times$$

$\times$ length $\times$ width $\times$ height of the tumor (mm^3)

Results — Conditioned media (CM) from three malignant cell lines (Sa 1, B 16, LLC) were tested for their influence on the adherence of normal non-stimulated peritoneal exudate MNC. The effect was compared to the influence of three CM, derived from non malignant cell lines (BHK, V-79,

kidney fibroblasts). Soluble tumor factors in the three CM from non malignant cell lines did not influence the adherence of MNC in the concentration range from 0 % to 80 % CM added to the cell suspension (Fig. 1). When compared to nonmalignant CM, CM from Sa 1 and LLC cell lines inhibited the adherence of the MNC. Adherence inhibition was concentration dependent and was observed in concentration 40 to 80 % CM. The B-16 cell-line-derived tumor products had minor influence on the

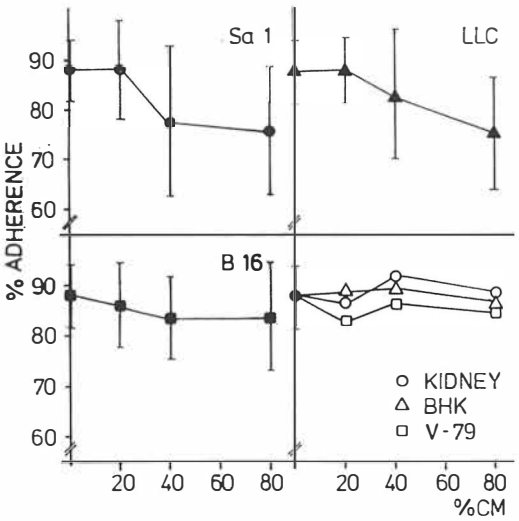

Fig. 1 — Adherence inhibition of peritoneal exudate MNC cells incubated for two hours in different concentrations of CM. CM were prepared from confluently growing cell cultures after 24 hours in serum free medium. Presented are results from four experiments in threeplicate as AM SD

Conditioned media (CM5)	% MAI AM ± SE
Sa 1	11.4 ± 3.3
LLC	13.3 ± 3.4
B 16	6.0 ± 3.5
BHK	0.2 ± 2.9
V-79	0.2 ± 2.5
Kidney fib.	0.6 ± 2.1

Table 1 — Influence of different CM on % macrophage inhibition (% MAI). Macrophages were incubated in 80% CM and the adherence inhibition calculated in relation to number of cells adhered in Eagle medium without CM. The results are presented as AM ± SE of four experiments in threeplicate

MNC adherence, its inhibition being in the range of CM derived from nonmalignant cell lines. Difference in the influence of CM from Sa 1 od LLC cell lines and B 16 derived CM was observed, the adherence inhibition of Sa 1 or LLC CM was significant ($p < 0.05$), whereas the B 16 CM was not. Also the percent of macrophage adherence inhibition (% MAI) was determined for the above mentioned CM at 80 % CM concentration. The presented results in Table 1 indicate that the strongest inhibition was observed with LLC derived CM, while the B 16 CM inhibited the adherence only to 6 %.

Since spreading of MNC and their migration are properties related to adherence, we investigated also the influence of CM on them. CM were tested for the influence on migration of normal MNC from glass capillary. Tumor factors of Sa 1, B 16 and LLC cell lines strongly enhanced the migration of MNC (expressed as migration index) compared to BHK and V-79 derived factors that inhibited the migration of cells. Surprisingly, CM from primary culture of kidney fibroblasts enhanced migration of MNC. Also in this experiment differences in the amount of enhancement between the malignant cell lines CM were noticeable, the strongest being Sa 1 CM (Fig. 2).

Enhancement of MNC migration caused by tumor derived factors was associated by spreading inhibition of these cells. MNC from healthy A/J mice were incubated for thre hours in cell culture conditions, non-adherent cells were washed away and the remaining cell culture incubated in 50 % CM. After 24^h the cells with elongated processes and rounded cells were scored. The tumor-cell-derived CM strongly inhibited spreading of macrophages, but no difference in spreading between the three malignant cell CM was observed (Fig. 3).

In vivo CM were tested for the influence on subcutaneously and intramuscularly growing tumors. CM were injected intravenosluy in order to test systemic effect of tumor factors on growth rate of autologous tumor. When CM were injected on the first and the second day after tumor transplantation, enhanced tumor growth rate was recorded (Fig. 4). The same effect was test-

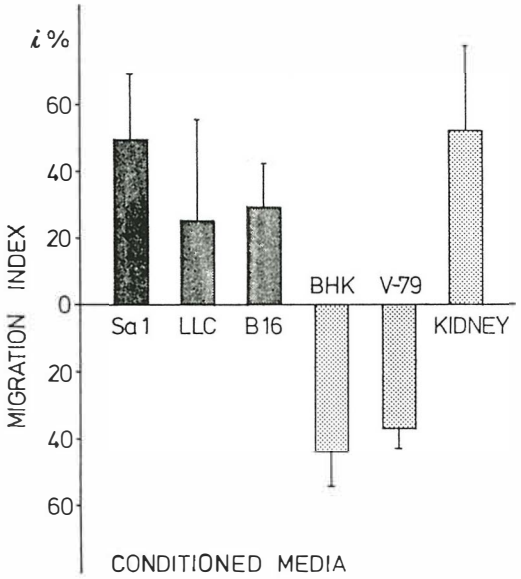

Fig. 2 — Migration index (i %) of peritoneal exudate MNC in different CM. The results are expressed as migration area of MNC in CM in relation to MNC incubated in EMEM. The columns represent arithmetic mean and the bars SD of two experiments in threeplicate

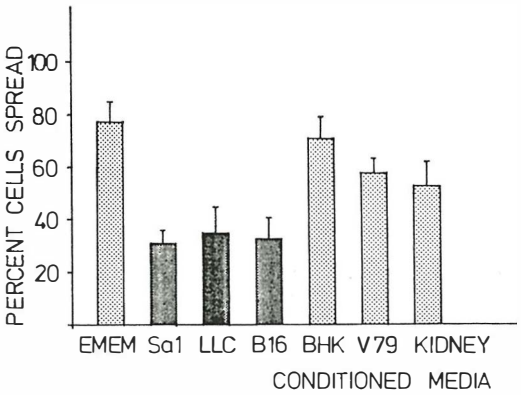

Fig. 3 — Percent of spread peritoneal macrophages was scored after 24 hours incubation in 50 % different CM. Spread cells with elongated processed were counted after staining with May-Grünwald Giemsa. Results from two experiments are presented as AM ± SD

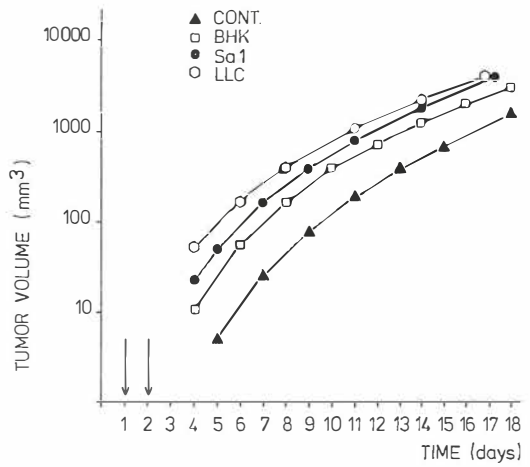

Fig. 4 — Growth curves of Sa 1 tumor after intravenous injection (arrows) of baby hamster kidney (BHK), sarcoma (Sa 1) and Lewis lung carcinoma (LLC) CM. In the control group cell culture medium was injected. The best fit curves were calculated from data by the least square method, each group of mice was comprising 8—10 A/J mice

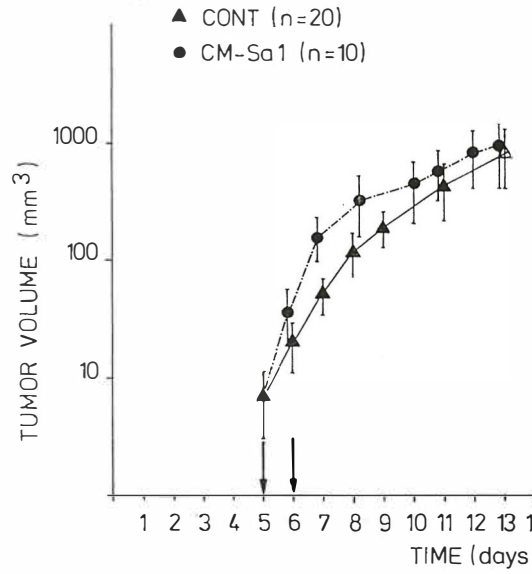

Fig. 5 — Growth curves of Sa 1 subcutaneous tumors after intravenous injection (arrows) of 0.2 ml Sa 1 CM. The tumor volumes are presented as AM ± 1 SD of the measurements

ed on already growing tumors, which reached the volume of 7 mm³. Tumor growth rate was accelerated after the first and the second day following intravenous CM injection (Fig. 5). Further, the growth promoting potential of Sa 1, LLC, BHK CM in comparison to medium control on subcutaneously growing Sa 1 tumor was tested (Fig. 4). Nonomalignant BHK CM promoted the tumor growth to almost the same extent

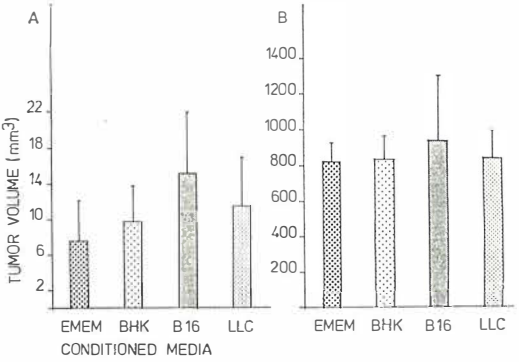

Fig. 6 — Volume of intramuscularly growing melanoma B 16 in C 57 B 1 mice 7 days (A) and 14 days (B) following tumor cell injection. The CM were injected intraperitoneally every second day after tumor transplantation. Each group comprised 10 mice, columns represent arithmetic mean, the bars standard deviation of the measurements

the autologous Sa 1 CM, whereas the LLC CM, a very aggresively growing tumor in vivo, promoted the growth to greater extent than Sa 1 CM. CM also influenced the survival of animals. The mice injected with control medium survived approximately 20 ± 3 days (AM $\pm$ SD), CM from malignant cell lines moderately shortened survival of the animals Sa 1 16 ± 2 days and LLC CM 16 ± 5 days, while the BHK CM prolonged mice survival to more than 25 days.

When CM were injected intraperitoneally into intramuscular melanoma B 16 bearing mice, similar effects as by Sa 1 subcutaneous tumor growth promotion was observed. Continuous application of (0.2 ml) B 16 CM intraperitoneally every second day promoted the tumor growth in the early phase, while in the late phase, when the tumor mass was big, no difference in tumor volume was observed. The LLC CM slightly promoted the B 16 tumor growth, however only in the early phase after tumor transplantation (Fig. 6).

Discussion — In the experiments CM containing tumor factors were prepared from tumor cell cultures in serum free media. It is known that specific and non-specific blocking factors have been identified, the specific ones comprising tumor antigens and antigen-antibody complexes, the non-specific ones comprising mainly small molecular weight proteins. In our experiments we demonstrated that CM prepared from various tumor cell lines contain factors that inhibit MNC adherence, stimulate their migration and reduce spreading These results are in accordance with the results of Cheung H. T. et al. (3) who has characterized a small molecular weight factor from murine tumors. Tumor factors from different tumor cell lines had influence on the adherence of MNC to various extent. The B 16 tumor products had minimal effect on adherence inhibition, while the Sa 1 and LLC CM reduced the adherence to much greater extent. This difference was observed also with migration stimulation of MNC, where the biggest influence was with Sa 1 CM, thought the spreading ability was reduced to almost the same extent with all three CM. These results might indicate that different tumor cell lines shed in CM nonspecific factors to various extent and that this may be a specific property of cell line. The tumor factors in vivo may influence the MNC properties either in the tumor itself or may be shed into the bloodstream and cause general immunosuppression, as determined by various authors.

The nonspecifity of these tumor factors has been tested also in vivo on subcutaneously growing sa 1 tumors. The Sa 1 and LLC CM both stimulated the tumor growth and shortened the survival of animals. When CM were applied systemically (intravenously) in small quantities, their action might be on the whole organism, and locally, in the site of the tumor growth. The promotion of tumor growth was observed also at the late phase of tumor growth resulting in the promotion already on the second day after CM application. The action of tumor factors may not be restricted only to the influence on MNC functions, but may be the result of multifaced interactions with immune cells and also influence on tumor and normal cells replication in tumor mass.

When CM were applied continuosly every second day into the abdominal cavity of the animal, the B 16 tumor growth stimulation was smaller. This might also be result of more difficult tumor diameter measurements and of more diffuse and less solid

tumor shape. Though the tumor growth was promoted with autologous B 16 CM in the early phase till the 7th day, the effect vanished on the 14th day after tumor inoculation. CM of heterologous LLC cell line had no measurable effect on the tumor growth promotion and in this case it could be speculated that either the route of application was not convinient or the growth promoting effect was not expressed on the other type of tumor as Sa 1.

In conclusion, we presume that tumor cell lines shed factors in culture media that nonspecifically influence in vitro properties of MNC to various extent, proposing that this is a cell line related property. On the other hand, these factors applied in vivo systemically promote the tumor growth. Enhancement of tumor growth was observede with either autologous or heterologous CM. The effect might be related to the tumor type observed, considering that LLC CM very expressively promoted subcutaneous Sa 1 tumor growth, but not intramuscularly growing B 16 melanoma.

Izvleček

Iz strnjeno rastočih celičnih kultur v mediju brez seruma smo pripravili kondicionirane medije (CM), ki vsebujejo topne tumorske produkte. CM treh različnih tumorskih linij so vplivali na tri sorodne lastnosti mononuklearnih celic (MNC): adherenco, migracijo in razširjanje. Avtologni CM Sa 1 in tudi heterologna CM melanoma B 16 in Lewis lung carcinoma (LLC) so zmanjšali imunske sposobnosti normalnih MNC A/J miši. Najmanjši vpliv je imel CM melanoma B 16 v primerjavi s CM Sa 1 in LLC. CM vbrizgani intravenozno ali intraperitonealno v živali so pospeševali rast avtolognih tumorjev. Nespecifičnost tumorskih faktorjev se je izrazila s pospeševanjem rasti heterolognih tumorjev. LLC CM so pospeševali rast sarkomskega tumorja po intravenoznem vbrizganju CM, dočim intraperitonealna apilkacija CM LLC ni imela tako velikega vpliva na rast mehanoma B 16 v nogi živali.

References:

1. Balm F. J., Blomberg van de Flier B. M. E., Drexhage H. A., Haan Meulmam M., Snow G. B.: Mononuclear phagocyte function in head and neck cancer: Depression of murine macrophage accumulation by low molecular weight factors derived from head and neck carcinomas. Laryngoscope 94: 223—227, 1984.

2. Botazzi B., Polentarutti N., Balsari A., Boraschi D., Ghezzi P., Salmona M., Mantovani A.: Chemotactic activity for mononuclear phagocytes of culture supernatants from murine and human tumor cells: Evidence for a role in the regulation of the macrophage content of neoplastic tissues. Int. J. Cancer 31: 55—63, 1983.

3. Cheung H. T., Cantarov W. D., Sundharadas G.: Characteristics of a low-molecular-weight factor extracted from mouse tumors that affect in vitro properties of macrophages. Int. J. Cancer 23: 344—352, 1979.

4. Gautam S. C.: Production of immunosuppressive factor(s) by a weakly immunogenic fibrosarcoma T 241. Anticancer Res. 3: 263—268, 1983.

5. Hellström K. E., Hellström I.: Enhancement of tumor outgrowth by tumor associated blocking factors. Int. J. Cancer 23: 366—373, 1979.

6. Hiragun A., Yoshida Y., Sato M., Tominaga T., Mitsui H.: Isolation of two syngeneic cell lines from a rat mammary carcinoma: Growth factor production by neoplastic epithelial cells. JNCI 75: 471—482, 1985.

7. Ianello D., Bonina L., Delfino D., Berlinghieri M. C., Mastroeni P.: Inhibition of normal rat macrophage function by soluble tumor products. Effects of systemic treatment with bacterial immunomodulators. Cancer Immunol. Immunother. 17: 38—41, 1984.

8. Kumary B. P., Vasudevan D. M.: Leucocyte adherence inhibition assay (LAI) in caner of the oral cavity. Eur. J. Cancer Clin. Oncol. 20: 891—897, 1984.

9. Pellis N. R., Yamagishi H., Shulan D. J., Kahan B. D.: Use of preparative isoelectric focusing in a sephadex gel slab to separate immunising and growth facilitating moieties in crude 3 M KCl exctracts of murine fibrosarcoma. Cancer Immunol. Immunother. 11: 53—58, 1981.

10. Putman J. B., Roth J. A.: Identification and characterization of tumor derived immunosuppressive glycoprotein from murine melanoma K-1735. Cancer Immunol. Immunother. 19: 90—100, 1985.

11. Rhodes J.: Resistance of tumour cells to macrophages. Cancer Immunol. Immunother. 7: 211—215, 1980.

12. Rogan Grgas J., Milas L.: Effect of tumor cell culture media and sera from tumor host on spreading, phagocytosis, and antitumor cytoxicity of C. Parvum-activated murine macrophages. Cancer Immunol. Immunother. 6: 169—173, 1979.

13. Saito H., Tomioka H.: Suppressive factor of tumor origin against macrophage phagocytosis of Staphylococcus aureus. Br. J. Cancer 41: 259—267, 1980.

14. Serša G., Krošl G., Rudolf Z.: The influence of different sera on the adherence and phagocytosis of mononuclear cells. Zdrav. vestn. 54: 49—53, 1985.

15. Serša G., Batista U., Krošl G., Rudolf Z.: Influence of soluble tumor products on growth of V-79 cells and methylcholanthrene carcinogenesis in mice. Period. Biolog. (in press., 1986).

Author's address: Gregor Serša, M. Sc., The Institute of Oncology, Zaloška 2, 61000 Ljubljana, Yugoslavia.