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Influence of Transplantable Melanomas on the Secretion of Cytokines by Hamster Peritoneal Macrophages

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Abstract. The subject of the study was the influence of two lines of transplantable melanomas on the secretion of cytokines (IL-6, TNF-α, OSM) and total proteins by hamster peritoneal macrophages. The results showed a statistically significant increase of total protein content and decrease of cytokine content in the supernatants of 24 h cultured macrophages from melanoma-bearing animals in comparison with control macrophages. Changes in the secretory function were more marked in the case of macrophages from hamsters bearing amelanotic melanoma. This may suggest that the biological features of melanomas can influence peritoneal macrophages to release the particular cytokines at different levels.

Key words: interleukin 6; tumor necrosis factor α; Oncostatin M; peritoneal macrophages; transplantable melanomas.

Introduction

The role of cytokines in tumor development and progression is at present a problem interesting a lot of investigators[2, 3, 15, 24, 34]. Not only have macrophages been shown to express a significant secretory function, including also cytokines, but also a tumoricidal function of these cells is generally approved[13, 14, 16, 29]. A number of reports have indicated that the activity of cytokines may have a prowerful local antitumor effect[3, 8, 9]. According to contemporary views, the secretion and activity of cytokines should be considered as an interdependent system that forms a network[7, 25, 26, 27]. According to the opinion of some authors, the pattern of autocrine regulation of these cytokines is as follows: interleukin 6 (IL-6) suppresses tumor necrosis factor α (TNF-α) secretion while IL-6 and Oncostatin M (OSM) evoke IL-6 production in both normal and neoplastic cells[7, 11, 33, 35, 37].

In continuation of our study of changes in macrophage reactivity induced by two lines of transplantable melanomas of the same origin but differing in growth rate, cell differentiation, antigenicity and immunogenicity[17, 18, 20], it was particularly interesting to find to what extent the activity of macrophages in animals bearing two melanoma lines was modified in comparison with the spontaneous secretion (without any activation) of control macrophages as regards the secretion of IL-6, TNF-α and OSM cytokines, whose participation in the regulation of tumor growth, against the general activity of macrophages, is especially stressed[24, 28, 32, 34].

Materials and Methods

Animals. Male Syrian (golden) hamsters, 2–3 months old, were used.

Transplantable melanomas. The tumors were transplantable melanotic (Ma) and amelanotic (Ab) melanomas. The melanotic line derived from a spontaneous melanoma of the skin which had appeared spontaneously in a breed of golden hamsters in 1959[6] and was de-

scribed by BOMIRSKI[5] as the Ma line. The amelanotic melanoma line originated from the melanotic from by a spontaneous alteration described in 1977 as the Ab line[5], in which loss of pigment was accompanied by an acceleration of growth[5], changes in antigenicity and immunogenicity[19].

The hamsters were injected with a suspension of melanoma tissue which was minced and mechanically dissociated in a glass homogenizer. Hamsters with transplanted melanotic melanoma were used for the experiments 21–24 days after the inoculation, and those with amelanotic melanoma 10–12 days after inoculation.

Macrophages – isolation and culture. Peritoneal exudate cells were induced by injection with 10 ml of 2.98% thioglycolate medium (Difco Inc. USA), and were washed out of the peritoneal cavity 5 days later by means of 0.9% NaCl. Then they were isolated by the method described previously[38]. The obtained cells consisted of 98% macrophages with 95–98% viability.

Macrophages of a concentration of 1×10^6/ml were incubated in RPMI 1640 (Gibco) (without FCS-fetal calf serum) for 1 h in 6-well plates (Corning) and then nonadherent cells were removed by washing with medium, so cytokine secretion was appreciated for adherent cells only. Then fresh medium was added and the cells were incubated for 24 h and, after that time, supernatants were harvested and stored at –70°C until later use. Peritoneal macrophages were harvested from animals with melanotic and amelanotic melanoma and from normal animals (control group).

Target cells for biological assays of cytokine activity:

– **B9** cell line (murine B cell hybridoma) sensitive to IL-6 was used.

– **L929** cells (transformed fibroblast of C3H mice) sensitive to TNF-α cytotoxicity were used.

– **A375** cells (human malignant melanoma) sensitive to OSM cytostatic activity were used (kindly provided by prof. J. Georgiades, Amarillo Cell Culture, USA).

Cytokine bioassays. IL-6, TNF-α, OSM bioactivity was measured by a colorimetric method with MTT (3--[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue; 5 mg/ml, Sigma)[30]. Samples were assayed in triplicate.

– **IL-6 bioassay**. The IL-6 bioactivity in macrophage supernatants was measured in a cell proliferation assay using B9 cells by a modification of the method of AARDEN et al.[1] The absorbance at 570 nm was determined in a BioRad microplate reader. The unit of IL-6 activity as defined as the supernatant amount required to cause a 50% increase of the cell number.

– **TNF-α bioassay**. TNF-α bioactivity in supernatants was determined by its cytotoxicity against actinomycin D (1 μg/ml) treated L929 cells by a modification of the method of OSTROVE and GIFFORD[31]. The unit of TNF activity as defined as the reciprocal of the dilution required to cause 50% cell destruction.

– **OSM bioassay**. OSM bioactivity in macrophage supernatants was determined by its ability to inhibit proliferation of A375 by a modification of the procedure described by LINSLEY et al.[22] The unit of OSM activity as defined as the volume fo supernatant required to cause a 50% inhibition of the growth of A375 melanoma cells.

Cytokine determination by ELISA assay. The level of IL-6, TNF-α and OSM in supernatants were determined by the Quantikine murine IL-6, TNF-α and human OSM immunoassays (Research and Diagnostic Systems, Mineapolis, MN, USA) which is a solid-phase ELISA. The assays were performed as described in the instructions. Absorbance at 450 nm was determined on a microplate reader (BioRad). Sensitivity limits of the ELISA for IL-6, TNF-α and OSM were 3.1 pg/ml, 5.1 pg/ml and < 6 pg/ml. Samples were assayed in triplicate.

Protein estimation. Total protein content in 24 h cultured macrophage supernatants was determined by the method of LOWRY et al.[23], using bovine albumin fraction V (Sigma) as a standard. Samples were assayed in triplicate.

Statistical evaluation. Group data expressed as mean ± SD were statistically estimated by Student's *t*-test. The p value less than 0.05 was considered to represent statistically significant differences.

Results

The results obtained, listed in Tables 1, 2 and Fig. 1, showed that hamster peritoneal macrophages *in vitro* secreted spontaneously, without any activation, proteins, among them cytokines such as TNF-α, IL-6 and OSM.

The secretory activity of the macrophages, determined by measuring the content of proteins secreted in 24-hour cultured macrophages, indicated that macrophages from animals bearing melanomas secreted more proteins than control macrophages (Table 1). Macrophages from animals with the transplantable melanotic melanoma line secreted about 45% more total proteins than the controls, and macrophages from hamsters bearing amelanotic melanoma as much as 65% more (the increase of secretion was statistically significant; Table 1).

The content of particular cytokines secreted after 24 h

culture varied and ranged from 385 pg/ml TNF-α secreted by control macrophages to only 12 pg/ml IL-6 secreted by macrophages bearing the amelanotic melanoma line (Table 2). The cytokine secreted in the largest amounts by macrophages of all groups was TNF-α. As in the case of the secretion of total proteins, the secretion of particular cytokines changed in animals with transplanted melanomas (Table 2).

Macrophages from control hamsters secreted about 385 pg of TNF-α per 1 ml, while macrophages from animals with melanomas secreted significantly less: Ma 291.4 pg/ml and Ab 188.9 pg/ml. The results of IL-6 content also showed that macrophages from hamsters with amelanotic melanoma secreted about 12 pg/ml, while control macrophages 26 pg/ml. Similar results were observed in OSM content, where control animals released 45 pg/ml, while those bearing melanomas about 30 pg/ml. This decrease is more pronouced if the cytokine content is calculated per 1 μg of the protein secreted (Table 2). After such calculation, we can see that macrophages from animals bearing the original Ma line secreted about 47.9% less TNF-α, 36,5% less IL-6 and 55.6% less OSM, while macrophages from animals with the Ab melanoma line secreted about 70.2% TNF-α, 72.6% IL-6 and 50% OSM less than the control macrophages (Table 2).

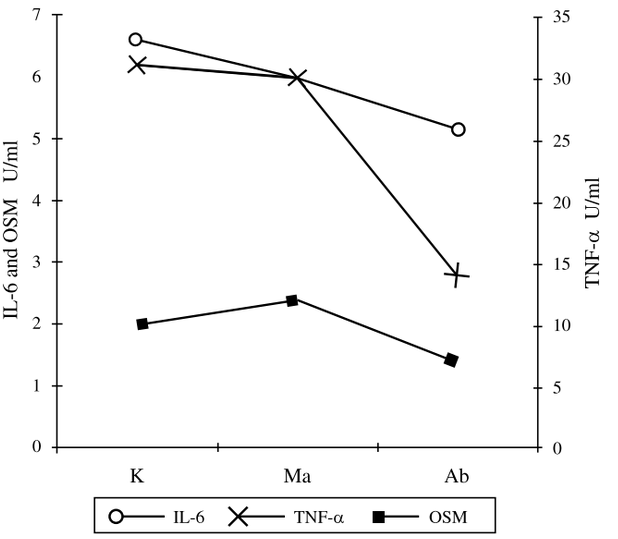

Fig. 1. IL-6, OSM and TNF-α activity in supernatants of 24 h cultured control (K) macrophages and those obtained from melanotic (Ma) and amelanotic (Ab) melanoma

Table 1. Protein content in supernatans of 24 h cultured control macrophages and macrophages from hamsters bearing transplantable melanomas

Supernatant of 24 h cultured macrophages of hamsters	Protein content expressed in μg/ml of supernatans	
	mean ± SD	p*
Control	126 ± 34	–
Bearing transplantable melanomas		
melanotic	183 ± 43	0.001<p<0.01
amelanotic	208 ± 35	0.001<p<0.01

The values are mean ± SD of 7 (for control macrophages), 9 (for macrophages of hamsters with melanotic melanoma) and 5 (for macrophages of hamsters with amelanotic melanoma) experiments done in triplicate. For each experiment 5 animals were used in each group.
* Student's *t*-test (p – in comparison with control macrophages).

The decrease of cytokine secretion (calculated per 1 μg of the protein secreted) was connected with the biological features of the melanomas, and in the case of TNF-α and IL-6 a markedly greater (about 70%) decrease was found for macrophages from animals bearing the Ab melanoma line, growing faster and with higher immunogenicity in comparison to melanoma line.

A similar tendency was observed in the biological activity of these cytokines, especially for TNF-α, whose activity in the control was 31 U/ml, while in amelanotic melanoma only 14 U/ml (statistically significant decrease; Fig. 1).

Table 2. IL-6, TNF-α, OSM content in supernatants of 24 h cultured control macrophages and those obtained from hamsters bearing transplantable melanotic (Ma) and amelanotic (Ab) melanoma estimated by ELISA test

Cytokine	Supernatant of cultured macrophages					
	control		from hamsters with melanoma			
			Ma		Ab	
	pg/ml	*pg/μg protein	pg/ml	*pg/μg protein	pg/ml	*pg/μg protein
IL-6	26.2 ± 13.2[1]	0.208	24.2 ± 13.5[2]	0.132	11.9 ± 3.1[3]	0.057
TNF-α	384.7 ± 120.4[4]	3.05	291.4 ± 120.3[5]	1.59	188.9 ± 66.7[6]	0.91
OSM	45.5 ± 9.9[7]	0.36	29.4 ± 6.0[8]	0.16	38.2 ± 11.4[9]	0.18

The values are mean ± SD of 13 (for IL-6), 9 (for TNF-α) and 4 (for OSM) experiments done in triplicate. For the experiment 3–5 animals were used in each group.
* Calculations of the cytokine content in 1 μg of the protein secreted by experimental group of macrophages. Student's *t*-test: 1 and 3 p<0.001, 2 and 3 p<0.005, 4 and 5 p<0.05, 4 and 6 p<0.001, 5 and 6 p<0.005, 7 and 8 p<0.05, 8 and 9 p>0.05.

Thus, with a generally intensified secretion of protein substances by peritoneal macrophages from animals bearing transplantable melanomas, the secretion of the studied cytokines decreased (Table 1, 2).

Discussion

As indicated by the results of the present investigations, the secretory activity of peritoneal macrophages from hamsters bearing transplantable melanomas is modified; at the same time our findings show in what a dramatic way, spontaneously, without restimulation *in vitro*, the macrophages reacted to the tumors present in the animals' bodies. The increase of protein secretion in the supernatants of the macrophage culture (from 50% in the case of animals bearing melanotic melanoma to 65% in animals bearing the emalanotic form) seems to show to what extent the dynamics of macrophage protein secretion is activated. Simultaneously, the decrease of the content of cytokines in the supernatants of the macrophages from animals bearing transplantable melanomas permits the assumption that these process are probably associated with the suppression of the genes for these cytokines.

Studies of the past decade have indicated the ability of neoplastic cells to modulated gene expression for cytokines[31, 37]. It is also noteworthy that the results obtained by us show how distinct the influence of the biological features of melanomas on the release of cytokines by macrophages is. The Ab melanoma line, with a greater immunogenicity, induced the release of the cytokines studied more distinctly than did its original counterpart. The cause of these changes may be numerous modifications in the plasmatic membrane of neoplastic cells, including transplantable melanomas, which can cause peritoneal macrophages to release different amounts of cytokines, which was mentioned in the reports of some authors and our earlier findings[10, 12, 19, 36].

The observed decrease of the release of the these cytokines, according to recent reports, should be considered in the aspect of a regulatory influence of these substances on the growth of melanomas and in the context of the cytokine network. It is known that IL-6 inhibits melanoma growth[32, 34], so it may be pressumed that the accelerated growth of the Ab melanoma line is connected, among other things, with the lower content of IL-6 secreted by macrophages of animals bearing this line of melanoma. However, the faster growth of the Ab melanoma line in comparison with the native Ma counterparts does not account for such a distinct decrease in the content of OSM, which acts as an inhibitor of melanoma growth and whose main source is macrophages[24, 28]. But a decrease in IL-6 and OSM secretion by macrophages of animals bearing melanomas might imply, in the biological sense, that melanomas suppress the production of cytokines which inhibit neoplasma growth (IL-6, OSM) and cytokines with a cytotoxic activity (TNF-α). However, when the correlation of the cytokines studied is considered in relationship to the cytokine network, we should say that the decreased TNF-α content, observed in this study, was accompanied by a decrease in the IL-6 secreted by the macrophages. This agrees with those reports which find that TNF-α induces IL-6 production[34]. A similar situation occurs in the case of OSM, because this cytokine is also able to stimulate IL-6 production[27]; therefore, we may suppose that the decrease of about 50% in its secretion by macrophages bearing melanomas is also the cause of the lower content of IL-6.

The biological implications of these phenomena are difficult to explain in detail, but the observations mentioned above may suggest that the development of melanomas in the body is connected with profound changes in macrophage immunobiology. Hence, it may also be assumed that a decreased secretion of macrophage factors enhancing immunobiological reactivity (IL-6, OSM), evoked by transplantable melanomas, is one more way of tumor escape from immunosurveillance.

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