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Estimation of macrophage IL-10 and NO secretion in the cytotoxicity against transplantable melanomas in relation to the progression of these tumours

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The relationship between the secretion of interleukin 10 (IL-10) and nitric oxide (NO) by hamster peritoneal macrophages and their cytotoxic effects on the cells of those two melanoma lines was studied.

The nonuniform reaction of macrophages from hamsters bearing two transplantable melanoma lines has been observed. An increase in the cytotoxicity of macrophages from hamsters bearing the amelanotic melanoma line was accompanied by an inverse correlation between IL-10 and NO secretion. Such a relationship was not found in the case of macrophages from animals bearing the native-melanotic melanoma line. It is suggested that the phenotypical changes of melanomas connected with their progression modified the cytotoxic and secretory activity of the macrophages with regard to IL-10 and NO.

key words: NO, interleukin 10, cytotoxicity macrophages, transplantable melanomas

INTRODUCTION

A number of reports have indicated that IL-10 can modulate antitumour immunological reactivity of macrophages [1, 8, 20, 26, 27]. Ealier observations underlined the fact that IL-10 produced by macrophages suppressed the immunological response to tumour [17, 19, 20, 22].

Most recent studies have shown that this cytokine is the inhibitor of the growth and metastasis of tumours [1, 9, 10, 15, 25].

It has also been described that IL-10 inhibits the production of many cytokines, i.e.TNF-α, NO which are involved in the cytotoxic reaction [21, 24, 27]. Moreover, it has been pointed out that although the pleiotropic action of IL-10 is known, its role *in vivo* in organisms bearing tumours has not been well explained [1, 25].

Our ealier investigations showed that the biological features of transplantable melanomas and their progression influenced changes in the cytotoxic activity of peritoneal macrophages [14]. Therefore, in a continuation of our study on changes in macrophage reactivity induced by two transplantable melanomas of the same origin but differing in the growth rate, cell differentiation [2, 4] and immunogenicity [11], it was particularly interesting to find out to what extent the cytotoxic effect of peritoneal macrophages is related to IL-10 and NO secretion. Also, it is advisable to establish if the IL-10 secretion by macrophages is modified in animals bearing two mela-

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noma lines in comparison with the secretion of this cytokine without any activation.

MATERIAL AND METHODS

Animals

Male Syrian (golden) hamsters, *Mesocricetus auratus* Waterhouse, 3–4 months old, were purchased from the Central Animal Facilities of the Silesian Medical University, Katowice, Poland. The animals were then conventionally reared at the Department's animal facility and fed standard diet and tap water *ad libitum*. The experimental procedures were approved by the Animal Ethics Committee at the Medical University of Gdańsk and conformed to the National Health and Medical Research Council's guide for the care and use of laboratory animals.

Transplantable melanomas

The tumours were transplantable melanotic and amelanotic melanomas. The melanotic melanoma line (Ma) derived from a spontaneous melanoma of the skin, which appeared spontaneously in a breed of golden hamsters in 1959. The amelanotic melanoma line (Ab) originated from the melanotic form by a spontaneous alteration into a more aggresive tumorogenic phenotype in which loss of pigment was accompanied by an acceleration of growth, a lower degree of differentiation [2, 4], changes in antigenicity and immunogenicity [11].

The hamsters were injected with a suspension of melanoma tissue obtained by mincing in a glass homogenizer. The tumour tissue was injected subcutaneously into the flank region in an amount of 200 mg of melanotic per hamster and 50 mg of amelanotic melanoma per hamster. 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. Differences in the quantity of transplanted tumours and the duration of time after inoculation were adjusted to the rate of growth of these two melanoma lines.

Animals consisting of 3–4 hamsters were used in each experimental group (hamsters with Ma and Ab line and normal animals).

Isolation of melanotic and amelanotic melanoma cells

Melanoma cells were isolated from solid tumours by a non-enzymatic method, described previously to obtain single-cell suspension [3]. The suspension consisted of 95–98% of viable cells estimated by test with trypan blue.

Macrophages

Peritoneal exudate cells were induced by injecting animals with 10 ml of 2.98% thioglycolate medium (Gibco), and five days later were washed out of the peritoneal cavity by means of 0.9% NaCl. They were then isolated by the method described previously [28]. Peritoneal macrophages were thus harvested from control and experimental groups.

Preparation of supernatants from macrophages

Macrophages at a concentration of 1×10^6/ml (for cytotoxicity assay) and 3×10^6/ml (for ELISA assay) were incubated in RPMI 1640 (culture medium, BioMed, Lublin) (without FCS-foetal calf serum) for 1h in 6-well plates (Sarstedt) and then nonadherent cells were removed, fresh medium was added and incubated for 24 h (for NO estimation) and 48 h (for IL-10 estimation and cytotoxic activity), respectively.

Assay of supernatant cytotoxic activity

Cytotoxic activity of supernatants against the cells of transplantable melanomas was measured by a colorimetric method with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (Sigma-Aldrich, 5 mg/ml PBS) [18]. Briefly, melanoma cells suspended in 500 μl of the medium [RPMI 1640 (BioMed Lublin, 10% FCS (GIBCO), antibiotics] at a concentration of 4×10^5/ml were incubated for 18 h with 500 μl of supernatants at 37°C. Cells incubated in medium without supernatants were used as a control sample.

Cytotoxicity of the supernatant was measured spectrophotometrically using absorbance at 570 nm.

% cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = 100 - \frac{\text{absorbance of samples with supernatants}}{\text{absorbance of samples without supernatants}} \times 100$$

IL-10 determination by ELISA test

Level of IL-10 in the supernatant of macrophages was determined by the Quantikine mouse IL-10 immunoassays (Research and Diagnostic Systems, Minneapolis, MN, USA) which is a solid-phase ELISA. The assay was performed as described in the instructions. Absorbance at 450 nm was determined on a microplate reader (BioRad). Sensitivity limit of the ELISA for IL-10 was 4.0 pg/ml. Samples were assayed in triplicate.

Assay of nitre oxide concentration

Nitric oxide (NO), quantified by the accumulation of nitrite (NO_2^-) (as a stable end product) in the 24 hr macrophage supernatants, was measured by a microplate assay method according to Ding et al. [7]. Briefly, 50 μl samples of each supernatant per well were transferred to a 96-well microassay plate (Nunc) and incubated with an equal volume of modified Griess reagent (Sigma-Aldrich) at room temperature for 10 min. Absorbance at 540 nm was determined in a BioRad microplate reader. Nitric oxide concentration was calculated from a sodium nitrite ($NaNO_2$, Sigma-Aldrich) standard curve. In all experiments nitrite contents in wells containing medium without macrophages were also measured and substracted from experimental values.

Data are expressed as nmol nitrite/1 $\times$ 10^6 cells/ml.

Statistical evaluation

Group data expressed as mean $\pm$ SD were statistically estimated by nonparametric Mann-Whitney U test by the STATISTICA program. The p value of less than 0.05 was considered to represent a statistically significant difference.

RESULTS

The results obtained regarding the relation between the IL-10 and NO contents in macrophage supernatants, and the cytotoxicity of these supernatants in control macrophages (from animals without melanomas, which have no contact with melanoma cells), and macrophages from animals bearing transplantable melanomas, are listed in Figure 1. We found a statistically significant increase (in comparison with control macrophages) of IL-10 content in the supernatants from macrophages of animals bearing melanotic melanoma and a statistically significant decrease of IL-10 secretion by macrophages from animals with amelanotic melanoma. At the same time the supernatant of control macrophages was found to contain the lowest amounts of NO when the concentration of IL-10 was at a medium level. In animals bearing transplantable melanomas the cytotoxic activity of macrophages increased and it was accompanied by changes in the content of the IL-10 and NO.

Statistical analysis of the results obtained concerning the cytotoxic activity indicated a statistically corroborated increase in the cytotoxic activity of supernatants from macrophages of animals bearing the amelanotic melanoma line.

The cytotoxicity was about 79%, while the cytotoxic activity of macrophages from animals with the native — melanotic melanoma line was 57% ($p < 0.001$, Fig. 1).

Simultaneously, we observed that macrophages from animals bearing melanotic melanoma showed a marked (in comparison with the control), 60% increase of IL-10 content and only about 18% higher secretion of NO.

On the other hand, after an amelanotic melanoma line transplantation an increase in macrophage cytotoxic activity was followed by a decrease in the secretion of IL-10, whose content in the supernatants was 45% lower than in the control macrophages and 66% lower than in macrophages from animals bearing melanotic melanoma, also with a statistically significant increase in NO secretion, which was 60% higher than in the control ($p < 0.05$; Fig. 1).

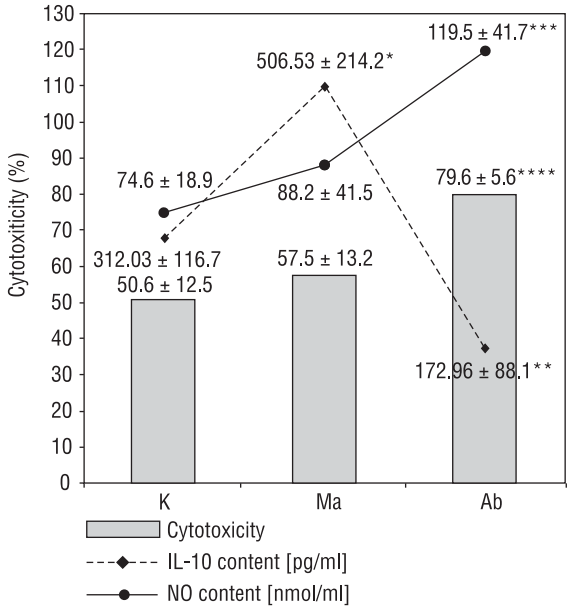

Figure 1. The cytotoxic activity of supernatants of control macrophages (K), obtained from hamsters bearing transplantable melanotic (Ma) and amelanotic (Ab) melanomas and IL-10 and NO content in these supernatants. The values are the means $\pm$ SD of 6–10 experiments done in triplicate. For each experiment 3–4 animals were used in each group. Statistical analysis by nonparametric Mann-Whitney U test: *statistically significant ($p < 0.05$) increase of IL-10 secretion by Ma macrophages in comparison with control macrophages, **statistically significant decrease of IL-10 secretion by Ab macrophages in comparison with control macrophages ($p < 0.05$) and Ma macrophages ($p < 0.001$), ***statistically significant increase of NO secretion by Ab macrophages in comparison with control macrophages ($p < 0.05$), ****statistically significant increase of Ab macrophages cytotoxicity in comparison with Ma macrophages ($p < 0.01$).

DISCUSSION

The results, indicating that macrophages from animals bearing two transplantable melanoma lines (compared with control macrophages) secrete different amounts of IL-10, seem to show that a spontaneous alteration of a native melanotic line into an amelanotic form accompanied by tumour progression is connected with changes in the secretion of IL-10 by peritoneal macrophages.

These results show that a changed phenotype and higher tumorogenicity of melanomas is connected with a statistically significant decrease of IL-10 secretion by macrophages. This may suggest that the secretion of IL-10 by macrophages depends on the biological features of melanomas. This observation is in agreement with our ealier investigations concerning the secretion of other cytokines by macrophages from animals bearing two transplantable melanoma lines [13].

Simultaneously during this study we observed that macrophages from animals with the amelanotic melanoma line with a higher growth rate, secreting less IL-10 had a higher cytotoxic activity against these melanoma line cells.

The decrease of IL-10 secretion by macrophages from animals with the amelanotic line could explain the higher cytotoxic activity of these macrophages as an effect of the decrease of the suppressor factor — IL-10 secreted by macrophages — the main type of cells producing this cytokine [6] .

This observation seems to agree with other studies, which suggest that IL-10 inhibits the antitumour activity of macrophages [16, 19, 21].

Simultaneously we observed a pronounced increase in NO secretion by macrophages from animals with amelanotic melanoma and the decrease in IL-10 secretion, which can explain the increase in the cytotoxic activity of these macrophages and on the other hand confirm the results of other authors, indicating that IL-10 inhibits NO production [21, 24, 26]; as our results indicate, the decrease of IL-10 secretion could be the cause of the higher NO secretion.

It should be underlined that this interpretation of the obtained results can refer only to macrophages from hamsters with the transplantable amelanotic melanoma line.

Although macrophages from animals bearing the native melanotic melanoma line, with a lower growth rate and a higher degree of differentiation, secreted more IL-10 and NO than the control macrophages, they did not increase significantly their cytotoxicity against these melanoma cells.

We may say that in the case of the melanotic line the IL-10 did not inhibit NO secretion because the increase of its content was accompanied by an increase in IL-10 secretion.

Therefore, the results mentioned above show that IL-10 does not always inhibit NO secretion. This observation is in agreement with Kundu et al. [16], who report that IL-10 does not inhibit NO production by melanoma cells but induces NO secretion and in this way inhibits the growth of these tumour cells. But such a relationship has not been described so far for macrophages.

Furthermore, our present investigations seem to indicate that peritoneal macrophages from animals bearing transplantable melanomas react nonuniformly to the kind of transplanted melanoma line and their cytotoxicity does not always depend on NO secretion.

In the interpretation of the biological implications of this problem Shearer's suggestion [23] — that there are subpopulations of macrophages which secrete cytokines enhancing the immunobiological properties of these cells, and subpopulations secreting IL-10 which suppress the immunological reaction — is helpful.

Bondenson et al. [5] also consider that the secretion by macrophages of cytokines inducing or inhibiting immunological reactions is regulated by different mechanisms. Besides, these authors suggested that even single macrophages can influence each other's cytokine secretion by paracrine or autocrine interactions.

Depending on the biological features of transplantable melanomas, the proportions of particular macrophage populations, including those secreting IL-10, might conceivably change. Our earlier study indicated changes in the heterogeneity of macrophages from hamsters with transplantable melanomas [11, 12].

We should also consider the results of other authors, who indicated that IL-10 can inhibit macrophage differentiation by enhancing their apoptosis [8, 26].

Thus, we could assume that macrophages from animals bearing the melanotic melanoma line, which secrete more IL-10, undergo apoptosis faster and that is why they are less active during the cytotoxic reaction than macrophages from animals with the amelanotic form, which secrete less IL-10. This problem still requires further studies.

To sum up the results, it may be concluded that the phenotype features of melanomas, connected with their progression, conspicuously modify the

secretory activity of macrophages as regards IL-10 and NO secretion.

We also observed that with melanoma progression the changed IL-10 secretion by macrophages was inversely correlated with the cytotoxic activity of macrophages, although it was not correlated with NO secretion.

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