Interrelationship between macrophage IL-12, IL-10 and NO secretion in expression of their tumoricidal activity

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Peritoneal macrophage activity in the secretion of IL-12, IL-10, NO and interrelationship between these substances in hamster bearing two lines of transplantable melanomas with different biological properties were studied.

It has been found that these macrophages lack of homogenous reactivity in the secretion of studied substances.

Melanotic melanoma line evoked moderate increase secretion of all above mentioned substances, whereas amelanotic melanoma line, tumor with higher immunogenicity, tumorogenicity and with shorter survival time of animals induced considerably increase of IL-12 and NO with prominent decrease of IL-10 secretion. Results allow to assume that macrophage secretory activity and interrelationship between these substances is not uniform in two lines of the same neoplasma and that it depends on tumor biological properties. Such observation seems to be useful in case of tumor cytokine immunotherapy.

Key words: macrophages cytotoxicity, transplantable melanoma, IL-12, IL-10, NO

Many studies indicate macrophages as the biggest population of cells producing IL-12 [3, 15, 41].

Among immunoregulatory cytokines IL-12 plays an essential role in the regulation of cell cytotoxicity, including macrophage antitumor activity [8, 11, 16, 36, 37] and cytokine network modulation [2, 12, 19, 27, 33, 38, 40].

It has been shown that IL-12 expression is regulated by NO [1, 26, 39], as well as by IL-10 [1, 2, 18, 26, 31] but tumor growth markedly decreases the production and activity of IL-12 secreted by macrophages [17, 30].

Investigations show that IL-12 induces IL-10 production by monocytes [12, 27] and IL-10 has the ability to limit IL-12 synthesis and activity [2, 28, 32], but the activity of endogenous NO synthase is necessary for the expression of the p40 subunit gene of IL-12 [34].

Moreover, because of IL-12 systemic and local action it has been suggested to use this cytokine in tumor therapy, including melanoma therapy [3, 15].

The influence of locally secreted IL-12 on tumor growth has been studied for almost ten years [10, 36].

As it is now known from our results [21, 22, 23, 25] and those reported by other authors [14, 30] that tumor growth changes the phenotype and function of macrophages, many scientists who consider these substances determining the tumor-host relationships are very important in tumor biology, focus their studies on the problem how tumors induce macrophages to produce immunoregulatory signals.

The interrelationships between the cytokines secreted by macrophages in organisms bearing tumors are still not fully understood [2, 35].

Continuing our studies on the influence of transplantable melanomas on the secretory activity of cytokines, NO and their antitumor action, we took into account the above reports as well as the fact that our earlier investigations had shown that the effector mechanism of macrophage cytotoxicity against two transplantable melanoma lines depends on the biological features of these melanomas [24]. Now it seemed reasonable to check if there is a relationship between IL-12 (immunoregulatory cytokine), IL-10 (immunosupressive cytokine) and NO secretion by peritoneal macrophages from animals bearing two transplantable melanoma lines – of the same origin but differing in growth rate, degree of differentiation – and the macrophage cytotoxic effect against cells of these two melanoma lines.

An understanding of the interactions between immunoregulatory and immunosupressive cytokines may possibly to

explain the tumor-host relationship and indicate a new approach to the use of cytokines in tumor immunotherapy.

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 Gdansk and confirmed by the National Health and Medical Research Council's guide for the care and use of laboratory animals.

Transplantable melanomas. The tumors 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 [4]. 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 [4, 5], changes in antigenicity and immunogenicity [20].

The hamsters were injected with a suspension of melanoma tissue obtained by mincing in a glass homogenizer. The tumor tissue was injected subcutaneously into the flank region in an amount of 200 mg of melanotic melanoma per one hamster and 50 mg of amelanotic melanoma per one 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 tumors and time of getting animals for experiments were adequate to the rate of growth of these two melanoma lines.

For the experiment 3–4 animals 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 tumors by a nonenzymatic method, described previously to obtain single-cell suspension [6]. The suspension consisted of 95–98% of viable cells estimated with trypan blue test.

Macrophages. Peritoneal exudate cells were induced by injection 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. Then they were isolated by the method described previously [42]. Peritoneal macrophages were harvested from animals with melanotic and amelanotic melanoma and from normal animals (control group).

Preparation of supernatants from macrophages. Macrophages at a concentration of $1x10^6$/ml (for cytotoxicity assay and NO estimation) and $3x10^6$/ml (for IL-10 estimation) and $4.5x10^6$/ml (for IL-12 estimation) were incubated in RPMI 1640 (culture medium, BioMed, Lublin) (without fetal 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 and IL-12 estimation and cytotoxic activity), respectively.

IL-10 and IL-12 determination by ELISA test. Levels of IL-10 and IL-12 in the supernatants were determined by the Quantikine murine IL-10 and IL-12 immunoassays (Research and Diagnostic Systems, Minneapolis, MN, USA) which is a solid-phase ELISA. The assay was preformed as described in the instructions. Absorbance at 450 nm was determined on a microplate reader (BioRad). The concentration of cytokines in the supernatants was determined by comparing the optical density of the samples to the standard curve (2–1500 pg/ml). Sensitivity limits of the ELISA for IL-10 and IL-12 were 4.0 pg/ml and 2.5 pg/ml. Samples were assayed in triplicate.

Western blot detection of IL-10 and IL-12. Fresh isolated macrophages ($2x10^6$) were lysated in hypotonic Tris/TritonX-100 buffer with protease inhibitor cocktail (aprotinin 100 μg/ml, leupeptin 100 μg/ml, iodoacetamide 1.8 mg/ml, phenyl-methyl sulphonyl fluoride (PMSF) at 1 mM) for 1 hour on ice, spun for 5 minutes at 10,000 rpm, supernatants were collected and stored at –70 °C until further processing. Proteins contained in the lysates were resolved by standard polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel. Separated proteins were then electroforetically transferred onto nitrocellulose (60 V, 2.5 h). Nitrocellulose sheets (Schleicher & Schuel) with transffered protein were then processed for IL-10 and IL-12 detection; blocked with 3% BSA in PBS, washed and exposed to the biotynylated anti-mouse IL-10 and biotynylated anti-mouse IL-12 (Research and Diagnostic Systems, Minneapolis, MN, USA) for 2 h at room temperature. After washing out the unbound antibody, the antibody bound to the membrane was detected by colorimetry using avidin-alkaline phosphatase solution (BioRad) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt)/ NBT (p-nitro blue tetrazolium chloride; BioRad) solution. Prestained SDS-PAGE Standards (BioRad) were used as molecular weight marker.

Assay of NO concentration. Nitric oxide (NO), quantified by the accumulation of nitrite (NO_2^-) (as a stable end product) in the 24 h macrophage supernatants, was measured by a microplate assay method according to DING et al [13]. 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 so-

dium 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/1x10^6 cells/ml.

Assay of supernatant cytotoxic activity. Cytotoxic activity of superntants against 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) [29]. Briefly, 500 μl isolated melanoma cells 4x10^5/ml in medium [RPMI 1640 (BioMed Lublin) 10% FCS (GIBCO), antibiotics] were incubated with 500 μl of supernatants at 37 °C for 18 h. Cells incubated in medium without supernatants were used as control sample.

Absorbance at 570 nm was estimated by a spectrophotometer.

% of cytotoxicity was calculated as follows:

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

Statistical evaluation. Group data expressed as mean ±S.D. were statistically estimated by nonparametric Mann-Whitney U test by STATISTICA program. The p value less than 0.05 was considered to represent a statistically significant difference.

Results

The results regarding the relation between the IL-10, IL-12 and NO contents in macrophage supernatants, and the cytotoxicity of these supernatants in control macrophages (from animals without melanomas, which had no contact with melanoma cells) and macrophages from animals bearing transplantable melanomas are listed in Figure 1 and Table 1.

Macrophages from animals bearing transplantable melanomas secreted more IL-12 and NO than control macrophages. This tendency is statistically significant for macrophages from animals with amelanotic melanoma line which secreted 60% more IL-12 and NO than control macrophages. Simultaneously these macrophages secreted about 45% less IL-10 (Tab. 1).

At the same time the supernatant of control macrophages was found to contain the lowest amounts of IL-12 and NO, when the concentration of IL-10 was at a medium level (Tab. 1, Fig. 1).

The presence of IL-10 and IL-12 was confirmed in the cellular lysates of macrophages from control and transplantable melanoma bearing animals by Western blot (Fig. 2).

Biologically active IL-12 as a 70kDa heterodimeric glycoprotein (p70) composed of a 40kDa subunit (p40) and a 35 kDa subunit (p35) was detected in the lysates of all the macrophages examined. Lysates of control macro-

Table 1. Interleukin 12 (IL-12), interleukin 10 (IL-10) and nitric oxide (NO) content in supernatants from the culture of peritoneal macrophages from control hamsters (K) and those bearing transplantable melanotic (Ma) and amelanotic (Ab) melanomas

Macrophages from hamster	Cytokines and nitric oxide content in culture supernatants		
	IL-12 (pg/ml) [4.5x10^6 cells/ml; after 48 h culture]	**IL-10 (pg/ml)** [3x10^6 cells/ml; after 48 h culture]	**NO (nmol/ml)** [1x10^6 cells/ml; after 24 h culture]
control	1.9 ± 0.29	312.03 ± 116.7	74.6 ± 18.9
bearing melanotic melanoma	2.62 ± 0.99	506.53 ± 214.2*	88.9 ± 41.5
bearing amelanotic melanoma	3.05 ± 1.17*	172.96 ± 88.1**	119.5 ± 41.7*

The values are the means ±S.D. for 3–10 experiments. Each experiment done in triplicate. Statistical analysis by nonparametric Mann-Whitney U test: *statistically significant increase of IL-12 by Ab macrophages, IL-10 by Ma macrophages, NO secretion by Ab macrophages in comparison with K macrophages ($p<0.05$); **statistically significant decrease of IL-10 secretion by Ab macrophages in comparison with K macrophages ($p<0.05$).

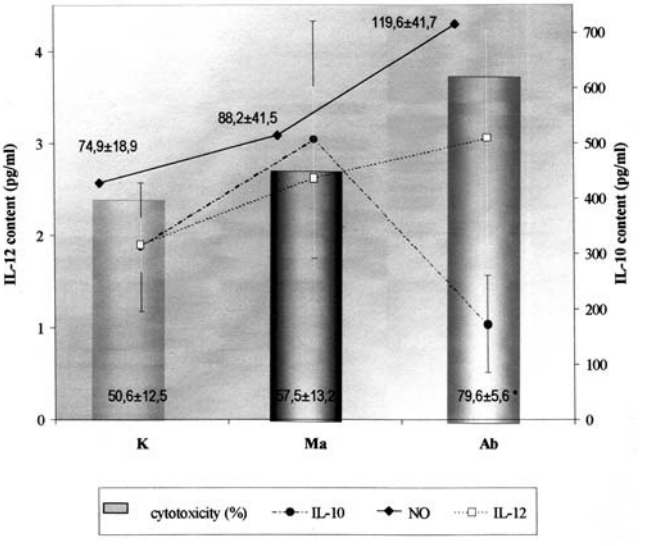
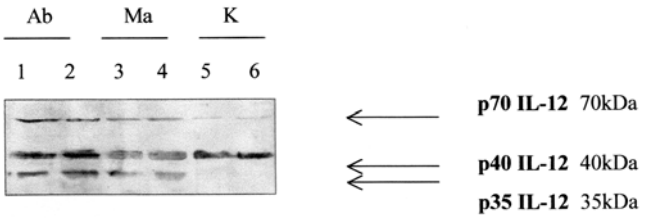


Figure 1. IL-10, IL-12 and NO content in supernatants from culture of peritoneal macrophages from control hamsters (K) and those bearing transplantable melanotic (Ma) and amelanotic (Ab) melanomas and the cytotoxic activity of these supernatants. The values are the means of 3–10 experiments. Each experiment done in triplicate. Statistical analysis by nonparametric Mann-Whitney U test: *statistically significant increase of Ab macrophages cytotoxicity in comparison with Ma macrophages ($p<0.01$).

A. IL-12

Lanes 1 and 2 are for cellular lysates of peritoneal macrophages from hamsters bearing amelanotic melanoma; lanes 3 and 4 for cellular lysates of peritoneal macrophages from hamsters bearing melanotic melanoma; lanes 5 and 6 for cellular lysates of peritoneal macrophages from control hamsters.



B. IL-10

Lane 1 is for cellular lysates of peritoneal macrophages from hamsters bearing amelanotic melanoma; lane 2 for cellular lysates of peritoneal macrophages from hamsters bearing melanotic melanoma; lane 3 for cellular lysates of peritoneal macrophages from control hamsters.

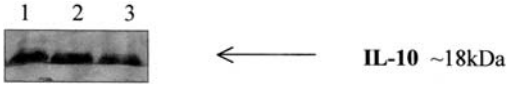


Figure 2. Western blot detection of interleukin 12 (A) and interleukin 10 (B) in cellular lysates of $2x10^6$ peritoneal macrophages from control hamsters (K) and those bearing transplantable melanotic (Ma) and amelanotic (Ab) melanomas.

phages had a lower p70 IL-12 expression than macrophages from animals bearing transplantable melanomas, while macrophages from amelanotic melanoma had more IL-12 than macrophages from animals with native melanotic melanoma line (Fig. 2A). There was no p35 unit in the control macrophages but the content of p35 and p40 units was higher in the lysates of macrophages from animals with amelanotic melanoma line (Fig. 2A).

IL-10 expression was similar in the whole macrophage group examined (Fig. 2B).

In animals bearing transplantable melanomas the cytotoxic activity of macrophages increased and was accompanied by changes in the content of IL-10, IL-12 and NO (Fig. 1).

Statistical analysis of the results concerning the cytotoxic activity indicated a statistically corroborated increase in the cytotoxic activity of supernatants of macrophages from 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% ($p<0.01$, Fig. 1).

Simultaneously, we observed that macrophages from animals bearing melanotic melanoma secreted about 60% for IL-10, 38% for IL-12 and only 18% for NO more than the controls (Fig. 1, Tab. 1).

On the other hand, after an amelanotic melanoma line transplantation we observed 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 IL-12 and NO secretion which was 60% higher than in the controls ($p<0.05$; Fig. 1, Tab. 1).

Discussion

We have found that macrophages from animals bearing two melanoma lines showed distinct differences in their secretory as well as tumoricidal activities.

The results confirm our earlier findings [21, 22, 23, 25] and data reported by other authors indicating that tumor growth alters macrophage phenotype and function [14, 30].

Besides, the present results are strong proof that the observed changes are determined by the biological properties of the melanoma lines studied.

In macrophages from animals bearing native melanotic melanoma line the secretion of IL-12 was higher by about 37% than in the control macrophages at a 60% IL-10 increase and a 20% NO increase.

The above-mentioned changes were accompanied only by a 7% increase in cytotoxicity of the macrophage supernatant.

Macrophages of animals bearing amelanotic melanoma line tumors with a more aggressive phenotype reacted by a 60% higher secretion of IL-12, a 45% decrease of IL-10 and a 60% increase of NO secretion. These changes were accompanied by an increase in macrophage cytotoxicity as high as 30%.

As there are reports that macrophages are the main source of IL-12 [15, 28, 41] and that the IL-12 tumoricidal activity is strong [3, 8, 10, 15], it may be supposed that the increased content of IL-12 in the supernatant of macrophages from animals bearing melanoma could also be responsible for the increase in their cytotoxity, in other words for the increased macrophage tumoricidal activity.

However, it should be underlined that not all authors agree that tumor growth increases IL-12 production by macrophages [17, 30].

When considering the interrelationship between macrophage IL-10 and IL-12 secretion in animals bearing two melanoma lines we did not find any inhibitory effect of IL-10 on the secretion of IL-12 by macrophages from animals bearing melanotic melanoma line. Such an effect was revealed in macrophages from animals bearing amelanotic line tumors with a more aggressive phenotype, where a decrease of IL-10 content was followed by an increase of IL-12 content. This does not agree with MUNDER et al report [32] that IL-10 inhibits the response induced by IL-12 and at the same time inhibits the production of IL-12 by macrophages.

This may indicate a lack of a distinct general regulatory influence of IL-10 on IL-12 secretion by macrophages or it could suggest that there exists some individual macrophage

reactivity dependent on the tumor biological properties, to which we drew attention in our earlier publications [21, 22, 23, 25].

BONDER et al [7] have postulated that the relationship between of IL-12 and IL-10 secreted by monocytes and macrophages in response to different stimulators depends on tissue specificity.

Some data also show that IL-12 can induce lymphocytes to produce IL-10 [27], which would indicate that there exists in these cells an intrinsic mechanism of a negative feedback limiting their activation.

Our observations on the presence of IL-12 and its subunits in the cytoplasm of macrophages from animals bearing melanomas, especially those from animals bearing amelanotic-melanoma, with an increased content of IL-12 and its subunits, in comparison with control macrophages, do not agree with data about a suppressive influence of IL-10 on gene expression for IL-12 subunits [34] but seem to be further evidence that the biological features of melanoma can modify the macrophage phenotype in a specific way.

Analysing our results concerning IL-10 and IL-12 interrelation in NO synthesis we could say that they are in agreement with the results of those authors who indicated that NO synthase is necessary for the expression of gene for subunit p40 of IL-12, and that NO autoregulates the expression of the IL-12 gene. But our results are not in agreement with the observation that peritoneal macrophages produced more IL-12 when NO synthase was inhibited [17, 31].

Our results seemed to indicate that IL-12 secreted by macrophages from animals bearing two transplantable melanoma lines induced the synthesis of NO.

Thus, by stimulating NO synthesis, IL-12 secreted by macrophages may be responsible for the increase of cytotoxic activity against the melanoma cells examined, especially against amelanotic melanoma cells.

In this way IL-12 can contribute to changes in the cytotoxic effector mechanism of macrophages, depending on the biological features of the melanoma line, as has been noted in our earlier publication [24].

It is worth to add that, as we have shown before [9], the observed increase in cytotoxic effect does not seem to be connected with changes in melanoma cell susceptibility to exogenous cytokines. From the results of this work it may be concluded that apart from NO also IL-12 secreted by macrophages from animals bearing amelanotic melanoma could be responsible for the significant increase in macrophage cytotoxicity against amelanotic melanoma cells.

To sum up, we could say that by modifying the secretory activity of NO, IL-12 secreted by macrophages from animals bearing melanoma inhances the antitumor activity of these cells but the effect is not uniform and depends on the biological features of the melanoma line examined. This should be taken into account when the use of IL-12 in antitumor therapy is considered.

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