Endogenous tumour necrosis factor-alpha sensitise melanoma cells to glucosaminylmuramyl dipeptide

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Abstract Flow cytometry was used to demonstrate that cultured human melanoma BRO cells expressed membrane-bound tumour necrosis factor-alpha (TNF-α) and were able to release TNF-α upon treatment with glucosaminylmuramyl dipeptide (GMDP). The released TNF-α was shown to prime melanoma cells, previously unable to respond to GMDP by increasing expression of melanoma-associated antigens, making them sensitive to GMDP treatment.

Key words: Tumour necrosis factor; Muramyl peptide; Melanoma-associated antigen

1. Introduction

Glucosaminylmuramyl dipeptide (GMDP) is known as potent immunomodulator which can cause inhibition of growth and necrosis of certain experimental tumours in mice [1]. We have reported earlier that the mechanism of GMDP anti-tumour activity, besides the activation of immune cells, involved induction of expression of tumour-associated antigens (TAAs), ICAM-1 and other antigens on tumour cells, in particular on human melanoma BRO [2–4]. The changes in the phenotype of melanoma BRO cells resulted in their increased lysis by peripheral blood cells of healthy donors [4]. Only a fraction of melanoma cells was able to respond to GMDP by increasing expression of melanoma-associated antigens, but the number of responding cells and the magnitude of the response increased considerably upon additional introduction of GMDP after 24 h of in vitro cultivation with this muramyl peptide [4,5]. In the present study we investigated the mechanism of this effect and have shown that TNF-α produced by melanoma cells was the priming agent.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal antibody (mAb) MCA-C1 (ATCC HB 8443) against melanoma-associated antigen (MAA) was kindly provided by Dr. E.S. Revazova (All-Russia Oncological Centre, Moscow), mAb 5A899 binding human IL-1β, by Dr. V.L. Yurin (State Research Centre 'Genetics', Moscow), and mAb E7H2 against human TNF-α [6], by O.G. Shamborant (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow). mAb reactive with TGF-β1β2β3 was purchased from Pharmingen, USA (Cat. No. 1835-01). rTNF-α was a gift of Dr. V.G. Korobko (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow). rTNF-α had specific activity $1–2\times10^7$ U/mg protein. It was more than 95% pure and contained less than 2 ng/mg TNF-α of endotoxin [7]. GMDP was synthesised as described in [8].

2.2. Cell culture

BRO and A-549 cells were cultivated as had been described in [4] in the mixture of Dulbecco's MEM/F12 Ham's medium (1:1), containing 10% foetal calf serum (FCS), 10 μg/ml gentamycin and 10 mM HEPES (complete medium). In several experiments cells were cultivated in serum-free DMEM/F12 Ham's medium, supplemented only with 10 mM HEPES.

2.3. Treatment with muramyl peptides

GMDP or MDP in water (10 μl) was added to 2×10^5 melanoma BRO or lung adenocarcinoma A-549 cells in 1 ml of complete or serum-free medium in 24-well plates. Incubation was carried out at 37°C. In control cultures water was substituted for muramyl peptide solution. When necessary second portion of muramyl peptide was added at 24 h. MCA-C1 expression was evaluated at 24 h after the second addition of muramyl peptide.

2.4. Trypsin digestion

Culture supernatant obtained by stimulation of BRO cells in serum-free medium was dialysed against phosphate-buffered saline (PBS), pH 7.4, for 18 h with one change of buffer. Trypsin in PBS was added to make final concentration 75 μg/ml, and the incubation was carried out for 4 h at 37°C. The sample was dialysed against incomplete medium. FCS was added (20 μl/ml) and TNF-α activity was evaluated by bioassay.

2.5. Heat inactivation

Culture supernatant was heated in boiling water bath for 1 min and dialysed against incomplete culture medium. FCS (20 μl/ml) was added, and the bioassay was performed.

2.6. Cytokine neutralisation assay

Anti-TGF-β1β2β3 (30 μg/ml), anti-IL-1β (100 μg/ml) and anti-TNF-α (80 μg/ml) mAbs were dissolved in PBS, pH 7.4. Serial dilutions of mAbs (1:5, 1:10, 1:20, 20 μl) were added to melanoma cell cultures (2×10^5 cells in 1 ml of complete medium) pre-treated for 24 h with GMDP (0.1 μg/ml). No mAb was added to control culture. GMDP (20 μg/ml) was added after 1 h incubation, and the incubation was continued for another 24 h. The percentage of MCA-C1 positive cells was evaluated by flow cytometry. The inhibition was calculated as follows:

$$\text{Inhibition } (\%) = \frac{\%\text{ of MCA–C1–positive cells in cultures without mAb} - \%\text{ of MCA–C1–positive cells in cultures with mAb}}{\%\text{ of MCA–C1–positive cells in cultures without mAb} - \%\text{ of MCA–C1–positive cells in control culture}} \times 100\%.$$

2.7. TNF-α bioassay

TNF-α bioassay was performed using TNF-sensitive L929 cells. Cytotoxicity was assessed by MTT test as in [9]. L929 cells in RPMI 1640 medium, containing 10% FCS and 0.5 μg/ml Actinomycin D (Sigma, USA), were placed into wells of 96-well microtitre plate

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Abbreviations: GMDP, *N*-acetylglucosaminyl-β1–4-*N*-acetylmuramyl-alanyl-D-isoglutamine; MDP, *N*-acetylmuramyl-alanyl-D-isoglutamine; TNF-α, tumour necrosis factor-alpha; TGF-β1β2β3, transforming growth factor-beta1,2,3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; mAb, monoclonal antibody; FCS, foetal calf serum; PBS, phosphate-buffered saline

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(4×10^4 cells in 180 μl medium per well). Twenty μl of rTNF-α (0.1–200 U/well) in complete medium or serial dilutions (1:10, 1:100, 1:1000) of dialysed supernatant from muramyl peptide treated BRO cells were added. The culture supernatant was dialysed against incomplete medium for 18 h with one change of medium and reconstituted before the assay with FCS to make 10% (v/v) final concentration. Incubation was carried out for 18 h at 37°C. MTT solution (0.5%, 10 μl) in PBS, pH 7.3, was added, and the incubation was continued for another 4 h. Supernatants were removed from wells, and cells were treated with 100 μl of 0.04 N HCl in isopropyl alcohol to dissolve formazan crystals. Optical density at 540 nm was assessed after 15 min incubation. Statistical analysis was carried out by Student's test using Statgraft program.

2.8. Flow cytometry

Staining of cells with FITC-labelled antibodies was performed as described by Valyakina et al. [2]. Briefly, cells were removed from the wells of 24-well plate with the aid of cell scraper, washed three times with PBS, pH 7.4, containing 2% FCS (PBS-FCS), and incubated for 30 min at 4°C with 100 μl of corresponding mAb solution in PBS-FCS, containing 10 μg mAb. After two washes with cold PBS-FCS, cells were treated for 30 min with FITC-labelled rabbit anti-mouse immunoglobulin antibodies (DAKO, Denmark, 1/100 dilution). Cells were washed 3 times with PBS-FCS, suspended in 1 ml PBS and analysed using EPICS-V flow cytometer, equipped with Spectra Physics laser (excitation wavelength 488 nm, power output 200 mV, barrier filter 515 nm). In each experiment at least ten thousand cells were analysed. Calculations were made with the aid of the MDADS software (Coultronics, France).

3. Results

In the previous study we have observed that treatment of tumour cells with GMDP resulted in initial (at 6 h) drop in the number of tumour-associated antigen expressing cells and the mean density of TAAs on cell surface followed by increase in these parameters with maximum at 24–48 h [2]. When the second portion of GMDP was added after 24 h the magnitude of the response increased considerably. In the case of melanoma BRO at optimal GMDP concentration the number of melanoma-associated antigen (MCA-C1 and MUC-18) expressing cells increased from 28% MCA-C1 and 13% MUC-18 positive cells to 45% and 29% respectively. Upon primary GMDP treatment the dose-response relationship had typical for immunomodulators bell-like shape. The MCA-C1 and MUC-18 antigen expression peaked at 0.1 μg/ml muramyl peptide concentration. The study of the dose-response relationship to second GMDP introduction revealed that substantially higher GMDP concentration (20 μg/ml) was required for optimal response (Fig. 1).

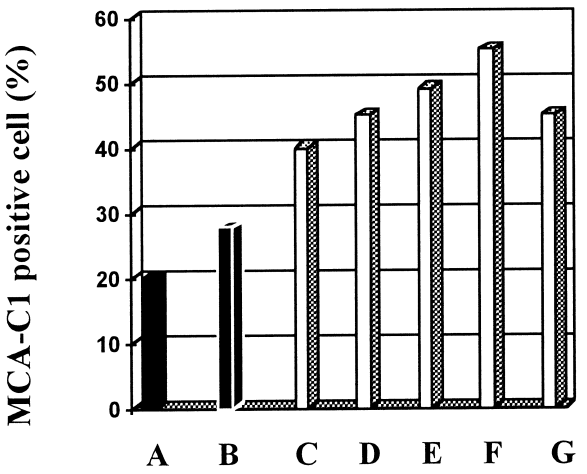

Fig. 1. Expression of MCA-C1 antigen on melanoma BRO cells cultivated in vitro with GMDP. A: Non-treated cells (control); B: single addition of GMDP (0.1 μg); C–G: double addition of GMDP with 24 h interval. First addition: 0.1 μg; second addition: C, 0.1 μg; D, 1 μg; E, 10 μg; F, 20 μg; G, 50 μg. MCA-C1 positive cells were counted using flow cytometry after fluorescent labelling with corresponding mAb 24 h after the last addition of GMDP. The data present the mean values of at least three separate analyses. S.D. does not exceed 10%.

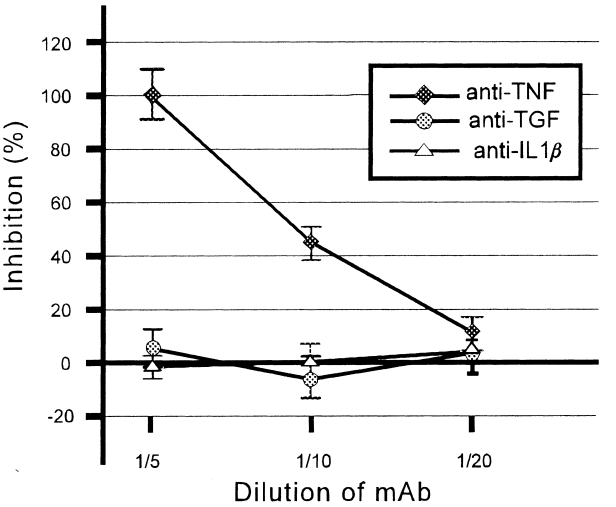

Fig. 2. Neutralisation of priming activity of supernatant of GMDP treated BRO cells with mAb against TNF-α, IL-1β and TGF-β. Serial dilutions of mAbs were added to melanoma cell cultures pretreated for 24 h with GMDP (0.1 μg/ml). GMDP (20 μg/ml) was added after 1 h incubation with mAb, and the incubation was continued for another 24 h. The percentage of MCA-C1 positive cells was evaluated by flow cytometry. Inhibition was calculated as described in Section 2.

The effect of GMDP priming on melanoma cells previously non-responding to muramyl peptide action was completely abrogated by change of culture supernatant for a fresh medium before second addition of GMDP. Moreover, addition of this supernatant to fresh melanoma cells along with optimum dose (20 μg/ml) of GMDP resulted in MCA-C1 expression characteristic for secondary GMDP response. It was noteworthy that melanoma cells had to be incubated with GMDP for at least 24 h before their conditioned medium acquired potency to prime intact cells.

The above results enabled us to assume the presence of cell priming factor in the conditioned medium of GMDP treated melanoma cells. The released factor was non-specific: the factor produced by melanoma cells was capable of priming lung adenocarcinoma A-549 cells and vice versa. In order to characterise this factor we performed GMDP stimulation in serum-free medium followed by trypsin treatment of supernatant. The loss of factor activity upon this treatment proved it to be a protein. The loss of factor activity was observed also upon heating. In contrast freezing-thawing resulted in considerable enhancement of factor activity. Evidently this could be due to dissociation of oligomeric factor molecules.

To identify GMDP priming factor we tried to neutralise the activity with monoclonal antibodies to known cytokines, namely TGF-β, IL-1β and TNF-α. Only mAb to TNF-α was able to completely inhibit priming activity of conditioned medium assuming that this cytokine was responsible for the effect (Fig. 2).

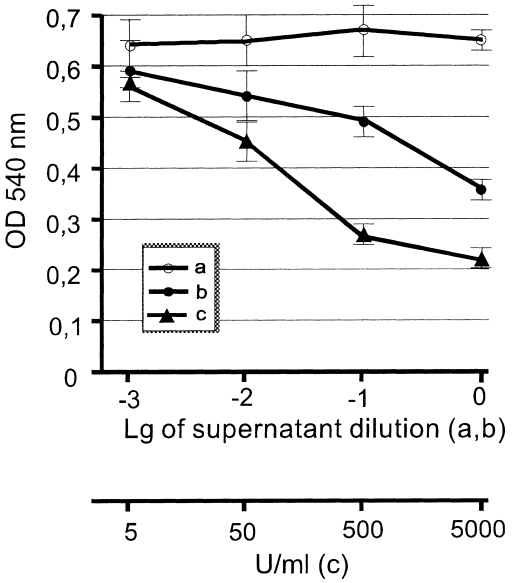

Fig. 3. Cytotoxic effect of culture supernatant from GMDP-induced melanoma BRO cells on murine L929 cells. a: Supernatant from cultured BRO cells (control); b: supernatant from GMDP-induced BRO cells; c: recombinant TNF-α. Cytotoxicity was evaluated using MTT test [9].

The presence of TNF-α in the condition medium from GMDP-induced melanoma cells was confirmed by biological test. Culture supernatant from growing melanoma cells was not cytotoxic to TNF-sensitive L929 murine fibroblasts whereas after 24 h GMDP stimulation the killing of L929 cells by the conditioned medium was observed (Fig. 3). GMDP remaining in supernatant (if any) could not by any means contribute to L929 killing, because it was removed by dialysis before the assay.

To prove the ability of TNF-α to sensitise BRO cells to GMDP treatment we used recombinant cytokine which was added to melanoma cell culture along with GMDP dose optimal for secondary response. Twenty-four h later MCA-C1 expression was evaluated. As can be seen from Fig. 4 recombinant TNF-α (100 U/ml) completely reproduced the effect of GMDP priming. The same result was obtained when TNF-α was added 2 h before the introduction of GMDP.

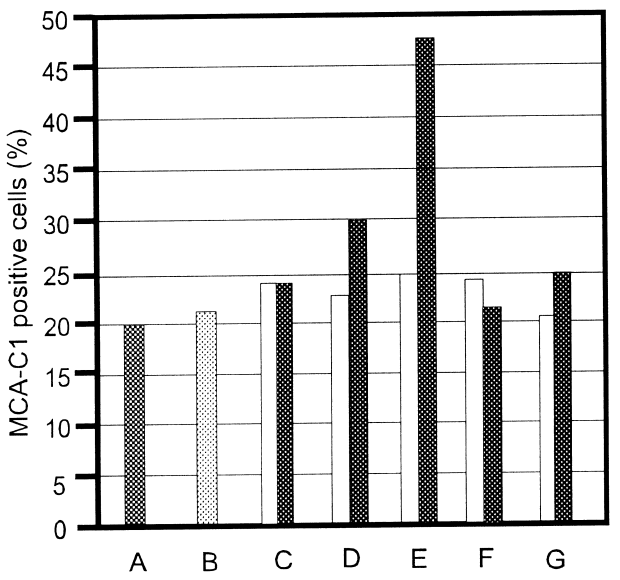

Fig. 4. Priming by rTNF-α of melanoma BRO cells to GMDP treatment. Cells were incubated for 24 h; A: without stimulators (control); B: with GMDP (20 μg/ml) alone; C–G: with (black bars) or without (white bars) GMDP (20 μg/ml) and various doses of TNF-α. C, 10 U/ml; D, 50 U/ml; E, 100 U/ml; F, 200 U/ml; G, 500 U/ml.

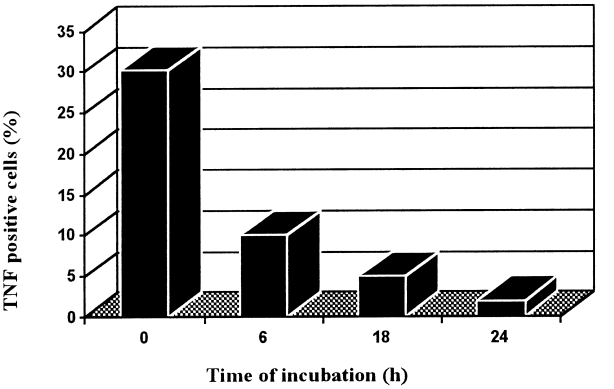

Fig. 5. Expression of TNF-α by melanoma BRO cells treated with GMDP (0.1 μg/ml) for 0–24 h. The percentage of TNF-α positive cells was evaluated by flow cytometry. The data present the mean values of at least three separate analyses. S.D. does not exceed 10%.

Flow cytometry was used to characterise the expression of TNF-α by BRO cells. Initially about 30% of cells had TNF-α on plasma membrane (Fig. 5). Treatment of cells with GMDP resulted in the release of TNF-α, and after 24 h incubation the cytokine could not be detected on cell surface. Evidently GMDP increased biosynthesis of TNF-α and induced its release from BRO cells.

In contrast to GMDP another well studied muramyl peptide, MDP, over wide concentration range (0.1–50 μg/ml) was not able to affect expression of MCA-C1 antigen and TNF-α on melanoma cells (data not shown), though it was known to induce TNF-α biosynthesis by macrophages [10]. When introduced together with TNF-α MDP was not able to augment MCA-C1 expression. Noteworthy MDP unlike GMDP was not capable of causing necrosis of experimental tumours [11].

4. Discussion

The results presented above demonstrate that stimulation of melanoma BRO cells with GMDP results in accumulation of TNF-α in condition medium. The production of this cytokine is not unique for melanoma BRO. Sander and Boeryd [12] have found that 12 out of 17 primary melanomas expressed TNF-α. It was found as well in other transformed cells where it performed the role of autocrine or paracrine growth factor [13,14]. The correlation of the TNF-α expression and metastatic potential of cancer cells was reported [15–17], namely, tumour cell-associated TNF-α induced expression of ICAM-1 and VCAM on vascular endothelium cells, facilitating high-affinity binding of tumour cells via β2-integrins and leading to tumour cell invasion.

In our hands TNF-α accumulating in culture medium was not cytotoxic to melanoma cells, but primed previously non-responding melanoma cells to subsequent GMDP treatment. The priming effect of conditioned medium from GMDP-stimulated lung adenocarcinoma cells on melanoma BRO

demonstrated that cytokine priming was not a unique feature of melanoma cells. That TNF-α was responsible for this effect was proved by neutralisation of supernatant priming activity with anti-TNF-α mAb as well as by priming of melanoma cells by rTNF-α.

The mechanism of TNF-α priming probably involved the exposure of new GMDP-binding sites by tumour cells. Various cytokines including TNF-α are known to induce expression of cell surface molecules [16]. Previously we demonstrated the presence of GMDP-binding sites inside BRO cells though the presence of low number of surface GMDP receptors was not ruled out (non-published results). The increase in number of membrane-binding sites on murine macrophages for another muramyl peptide, namely, MDP-stearoyl-lysine on murine macrophages upon cytokine priming was reported earlier for Ifn-γ [18]. The high dose of GMDP required for secondary GMDP response might reflect the lower affinity of newly expressed binding sites on induced BRO cells. The alteration of the affinity of MDP-binding sites upon cell activation was reported earlier by Silverman et al. for murine macrophages [19].

Not only GMDP, but other muramyl peptides as well are known to co-operate with TNF-α. The enhancement of IL-6 gene expression was observed upon simultaneous introduction of MDP and TNF-α into human monocytes culture [20]. Cooperative effect was also characteristic for these compounds in the induction of host resistance to bacterial infections [21]. Hence, taking in account that muramyl peptides induce TNF-α biosynthesis, the data obtained thus far points out that TNF-α might be involved in various activities of muramyl peptides, in particular, in anti-tumour activity of GMDP and, possibly, of other muramyl peptides. These immunomodulators seem to synergise activating immune cells and modify the phenotype of tumour cells. Besides, the priming effect of TNF-α on tumour cells might explain the earlier established fact that GMDP caused necrosis of only well developed tumours [8]. The reason for this was not established, but evidently only at the late stage of tumour development enough TNF-α is released for tumour cell priming.

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