An endogenous melanocyte-inhibiting tripeptide pyroGlu-Phe-GlyNH₂ delays *in vivo* growth of monoclonal experimental melanoma

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Abstract. The melanocyte-inhibiting tripeptide (MTP) pyroGlu-Phe-GlyNH₂ is present in tissue cultures of non-transformed melanocytes and melanoma cells and influences melanocyte growth *in vitro*. The objective of the present study was to investigate a possible effect of MTP on the *in vivo* growth of B16A2, a monoclonal experimental melanoma. The B16A2 clone was established by the limited dilution technique. It has a reduced DNA content and displays slower growth both *in vivo* and *in vitro* compared to the parent cell line (B16). B16A2 cells were injected subcutaneously into hairless mice at four sites (300 000 cells in 0.25 ml buffer/site). MTP was given by i.p. injection 3 times a week at two concentrations (1 pmol and 1 nmol/animal). The control animals received the equal volume of solvent. The animals were sacrificed 1 and 2 weeks after tumour transplantation, and all tumours were weighed. One week after transplantation, the animals who received 1 pmol MTP had fewer tumours and a reduced tumour load. Two weeks after the transplantation, the differences between control and treated animals were no longer observed. The results indicate that MTP temporarily delays *in vivo* tumour growth.

INTRODUCTION

Cell proliferation in normal and malignant tissues is regulated by the interactive network of growth-stimulating and growth-inhibiting molecules (Baserga, Porcu & Sell 1993, Epifanova & Brooks 1994, Johnson 1994, Marshall & Lord 1996). A group of endogenous growth-regulating oligopeptides identified in different cell populations during the past decade, seems to be important in negative feedback signalling (Guigon & Bonnet 1995, Elgjo, Reichelt & Gembitsky 1998). All these peptides consist of 3–5 amino acid residues, have a substituted *N*-terminus that makes them more resistant to aminopeptidases, a very low optimal active dose range (usually picomolar doses when given either *in vivo* or *in vitro*), bell-shaped dose–response curves, and a distinctive cell/tissue preference (Elgjo 1993, Elgjo *et al.* 1998). Recently, a new member of this family was identified, a melanocyte-inhibiting tripeptide

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(MTP) (Gembitsky *et al.* 1998b). MTP (pyroGlu-Phe-GlyNH₂) is present in cultures of non-transformed melanocytes and malignant melanoma (B16 cells). MTP causes transitory delay of cell growth in cultured non-transformed melanocytes resulting from a reversible inhibition of melanocyte transition from the G₂-phase of the cell cycle into mitosis (Gembitsky, Reichelt & Elgjo 1998). The biological activity of MTP *in vivo* has not been yet examined.

Malignant melanoma imposes a considerable public health burden (Stadelman & Reintgen 1998). Despite extensive research in this field, the molecular alterations responsible for the development and stepwise progression of melanoma are not fully understood (Meier *et al.* 1998). Interestingly, the amount of MTP was found to be substantially lower in B16 melanoma cells compared to normal melanocytes (Gembitsky *et al.* 1998b). B16 melanoma cells display extremely aggressive growth when transplanted into hairless mice. Usually, the animals have to be sacrificed 1 week after transplantation as a result of rapid tumour growth. This fact makes it impossible to carry out experimental treatment of tumour-bearing animals for a longer time. To perform *in vivo* experiments, a slower growing clone (B16A2), derived from wild-type B16 cells, has been established. The objective of the present study was to investigate a possible effect of MTP on the *in vivo* growth of B16A2.

MATERIALS AND METHODS

Chemicals

Melanocyte-inhibiting tripeptide pyroGlu-Phe-GlyNH₂ was custom synthesized by Peninsula Laboratories Inc. (Belmont, CA, USA). The peptide was stored dry at -70°C until used. Fetal calf serum and other reagents for cell cultures were obtained from Gibco BRL Life Technologies (Paisley, Scotland). Other chemicals were from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture

B16 melanoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown as a monolayer in Eagle's minimum essential medium supplemented with L-glutamine (2 mM), non-essential amino acids (2% v/v 100 × stock solution) and heat-inactivated fetal calf serum (7%). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Limiting dilution cloning of B16 cells

B16 cultures from a confluent flask were seeded at 0.5 cells/well in 96-well microtitre plates (Becton Dickinson Labware, Bedford, MA, USA) in 0.2 ml/well. The cells were allowed to attach to the substrate overnight and wells containing a single cell were marked. The clones were expanded using the cell culture protocol described above. The slowest growing clone was picked up and designated as B16A2.

Estimation of cell growth

The cells of either wild-type or clonal melanomas were seeded into 24-well plates (Corning Costar Co., Cambridge, MA, USA) at an initial density of 5300 cells/cm² in 2 ml culture medium. Exchange of culture media with a fresh one was carried out every second day. Determination of cell number was performed by trypsinizing cell cultures and counting the cells with a Coulter Counter[®] Z2 (Beckman Counter Inc., Fullerton, CA, USA) 24, 48, 72 and 96 h after seeding.

DNA flow cytometry

Tumour samples were minced in phosphate-buffered saline (PBS) and then fixed in absolute methanol for 1 h at -20°C . Samples were centrifuged at 1000 r.p.m., and then washed once with PBS. The cells were stained for DNA using the method of Vindelöv, Christensen & Nissen (1983) and propidium iodide (PI). The tumour samples were analysed for DNA content using a FACSVantage laser flow cytometer (Becton Dickinson, San Jose, CA, USA). The laser excitation wavelength was 488 nm and PI red fluorescence (DNA content) was collected as a liner measurement. Doublet discrimination and exclusion of debris and clumps were achieved using red fluorescence pulse-width analyses. DNA indices (DIs) were calculated from the peak values of the tumour G_0/G_1 peak and the diploid reference peak using Multiplus software (Phoenix Flow Systems, San Diego, CA, USA). Tumours with a $DI = 1.0$ were defined as DNA diploid, and those with a $DI > 1.0$ as DNA aneuploid; those with a $DI = 2.0$ were DNA tetraploid. The coefficients of variation (CVs) of the tumour G_0/G_1 peaks were less than 5.0%. Cell cycle analysis (calculation of percentage G_1 , S, G_2) were performed using Multiplus software.

In vivo experiments

Female *hr/hr* hairless mice, 6–8-week-old were obtained from Bommice Ltd. (Ry, Denmark). The animals were kept six to a cage in a room with a 12-h light–darkness rhythm (artificial light from 7 a.m. to 7 p.m.) with controlled temperature and humidity; they were given a standard diet and water *ad libitum*. The cages were cleaned and fresh water was supplied at noon three times a week. B16A2 cells were propagated *in vitro*, grown until subconfluence, trypsinized and resuspended in phosphate-buffered saline (PBS). The cells were injected subcutaneously into hairless mice on four sites at the back (300 000 cells in 0.25 ml PBS/site). MTP was dissolved in 0.1 mM acetic acid and given to animals by i.p. injections three times a week at two concentrations (1 pmol and 1 nmol/animal). Control animals received the equal volume of the solvent. The injections were given by using a ‘blind’ method. Each group of mice receiving either 1 pmol or 1 nmol MTP, contained 12 animals. Each control group consisted of 18 animals. The animals were sacrificed 1 and 2 weeks after the tumour transplantation, and all tumours were weighed. The following four variables were determined: (1) number of tumours per animal; (2) total tumour load – the total weight of all tumours of a single animal; (3) relative tumour load – weight of tumours calculated as % of body weight; (4) average weight of a single tumour. The experiments were repeated four times.

Statistical analyses

Statistical analyses were performed using two-tailed Mann–Whitney tests of significance. GraphPad Prizm software (GraphPad Prizm Inc., San Diego, CA, USA) was employed. Differences were recognized as significant at $P \leq 0.05$.

RESULTS

Characterization of B16A2 clone

Using the limiting dilution technique it was possible to derive several clones from wild-type B16 melanoma cells (parent cell line). The clones differed by their growth parameters. They displayed either a higher or a lower rate of proliferation compared to the parent cell line. The slowest growing clone was designated B16A2 and was further investigated. *In vitro* growth curves for B16 and B16A2 cells are shown in Figure 1. B16A2 cells displayed significantly

Figure 1. *In vitro* growth curves of B16 and B16A2 melanomas. Bars indicate 1 SEM.

slower *in vitro* growth compared to that of B16. At the stage of exponential growth (days 1–3), the doubling times for B16 and B16A2 cells were 13 and 14 h, respectively. When confluence was achieved, both cell lines continued to proliferate (days 3–4). At this stage their growth curves had a similar slope (Figure 1). On days 3 and 4 after the seeding the two growth curves differed significantly by 100 000 cells ($P = 0.0046$ and $P = 0.023$, respectively). The B16A2 clone also displayed slower growth when transplanted into hairless mice. The *in vivo* progression of B16A2 melanomas was approximately 1 week belated compared to that of B16 melanomas.

Cultured B16 and B16A2 cells had different DNA indices as determined by flow cytometry measurements of DNA content. The B16 parent cell line was DNA tetraploid with a DI of nearly 2.0, whereas the B16A2 clone was highly DNA aneuploid with a DI of 1.86. Cell cycle analyses of log-phase B16 and B16A2 cultures revealed that the B16 clone had a smaller S phase fraction than the parent B16 line (Figure 2, Table 1).

Estimation of anti-proliferative activity of MTP *in vivo*

B16A2 clone was used in all *in vivo* experiments. Tumour-bearing mice were treated with MTP three times a week (Monday, Wednesday, Friday). The first treatment was given immediately after injection of tumour cells. Since MTP has very low dose optima (10^{-11} M and lower) when tested for inhibition in tissue cultures of non-transformed melanocytes (Gembitsky *et al.* 1998a), two concentrations of MTP were chosen for the *in vivo* experiments (1 pmol and 1 nmol).

One picomole of MTP per animal per treatment inhibited tumour growth 1 week after tumour transplantation. The animals receiving this dose of MTP had fewer tumours, reduced

Figure 2. DNA content histogram of B16 (a) and B16A2 (b) melanoma cells

total and relative tumour load, and decreased average weight of a single tumour (Figure 3). The differences between treated and control animals were statistically significant (Table 2). Two weeks after transplantation the tumours were drastically enlarged and statistically significant differences between the control animals and the animals receiving MTP were no longer observed (Table 2). The dose of 1 nmol MTP per animal per treatment was ineffective after either 1 week or 2 weeks of treatment (Table 2).

DISCUSSION

Uncontrolled cell proliferation in tumour tissues may result from either increased sensitivity of cells to autocrine-acting mitogens or a failure to respond to autocrine growth inhibitors (Sporn & Roberts 1985). Endogenous growth-inhibiting peptides may therefore interfere with deregulated melanoma growth. This study investigated the anti-proliferative effect of MTP (pyroGlu-Phe-GlyNH₂) in mice with transplants of experimental melanoma B16A2.

B16A2 possesses a reduced DNA content and smaller fraction of cells in the S phase compared to the parent cell line (B16). It can therefore be concluded that during the cloning procedure a cell which lost some genetic material, has been selected and propagated. This appears to produce a clone that grows slower than the parent tumour. B16A2 was indeed shown to display a lower rate of proliferation compared to B16 when investigated both *in vivo* and *in vitro*.

MTP displays anti-proliferative activity *in vivo* at a low dose (1 pmol/animal/treatment). A 1 nmol dose was ineffective. This is in agreement with the bell-shaped dose-response curve

Table 1 Determination of the cell cycle distribution in B16 and B16A2 cells

Cell line	G ₁ (%)	S (%)	G ₂ + M (%)
B16	69.4	24.9	5.7
B16A2	86	6.8	7.2

Figure 3. Influence of MTP on the *in vivo* growth of B16A2 melanoma.

that has been established for MTP in tissue culture of non-transformed melanocytes (Gembitsky *et al.* 1998a). MTP delays *in vivo* tumour growth only temporarily, since 2 weeks after melanoma transplantation the differences between control and treated animals were no longer observed. Similarly, MTP provokes only a transitory delay of melanocyte growth *in vitro* (Gembitsky *et al.* 1998a). Other endogenous inhibitors have been reported to display transitory effects on cell proliferation. Thus, *in vitro* desensitization of human melanoma cells to transforming growth factor- β was demonstrated by Krasagakis *et al.* (1999). Also, after 2 weeks' treatment with endostatin, resumption of growth of human renal carcinoma in nude mice was observed (Yamaguchi *et al.* 1999).

The reasons for the transitory anti-proliferative effect of MTP *in vivo* are unclear. The peptide could force a selection of a subclone of melanoma cells which would be insensitive to the negative feed-back signals (e.g. selection of a subclone which does not express a putative MTP receptor). Alternatively, MTP could induce a proteolytic enzyme in melanoma cells (or facilitate selection of a subclone expressing such an enzyme) and in this way accelerate its own degradation.

The properties of MTP when administrated *in vivo* are similar to those of other endogenous growth-inhibiting oligopeptides that have been identified in several cell populations. This peptide family includes: epidermal pentapeptide (EPP), pyroGlu-Glu-Asp-Ser-GlyOH (Elgjo & Reichelt 1984), haemoregulatory peptide (HP5b), pyroGlu-Glu-Asp-Cys-LysOH (Paukovits & Laerum 1982), growth inhibitory hepatic peptide, pyroGlu-Gln-Gly-Ser-AsnOH (Paulsen, Reichelt & Petersen 1987), colon mitosis inhibitor (CMI), pyroGlu-His-GlyOH (Skraastad *et al.* 1987), and haemopoietic inhibitor (seraspenide), N-Acetyl-Ser-Asp-Lys-ProOH (Lenfant *et al.* 1989). A similar low-molecular weight factor of peptidic nature has been found in Ehrlich ascites carcinoma cells (Gembitsky *et al.* 1996, 1997). All these peptides exhibit their anti-proliferative effects at very low doses and have a restricted

Table 2 Influence of MTP on the *in vivo* growth of B16A2 melanoma (the values are given as mean $\pm$ SEM)

Variables	1 week after tumour transplantation			2 weeks after tumour transplantation		
	Control animals	MTP treated animals		Control animals	MTP treated animals	
		1 pmol	1 nmol		1 pmol	1 nmol
Number of tumours	3.33 $\pm$ 0.21	1.5 $\pm$ 0.42 <i>P</i> = 0.0087	2.33 $\pm$ 0.49 <i>P</i> = 0.132	2.83 $\pm$ 0.44	3.25 $\pm$ 0.35 <i>P</i> = 0.289	2.92 $\pm$ 0.39 <i>P</i> = 0.771
Total tumour load (mg)	144 $\pm$ 19	26.4 $\pm$ 8.0 <i>P</i> = 0.0043	149 $\pm$ 50 <i>P</i> = 0.937	1501 $\pm$ 617	1158 $\pm$ 250 <i>P</i> = 0.729	1073 $\pm$ 315 <i>P</i> = 0.751
Average weight of a single tumour (mg)	42.8 $\pm$ 5.0	19.4 $\pm$ 8.0 <i>P</i> = 0.05	52 $\pm$ 16 <i>P</i> = 0.699	419 $\pm$ 116	316 $\pm$ 67 <i>P</i> = 0.840	324 $\pm$ 76 <i>P</i> = 0.840
Relative tumour load (% of the body weight)	7.26 $\pm$ 0.96	1.26 $\pm$ 0.39 <i>P</i> = 0.043	8.31 $\pm$ 2.9 <i>P</i> = 0.990	60.6 $\pm$ 22.0	54.7 $\pm$ 11.8 <i>P</i> = 0.773	49.0 $\pm$ 14.1 <i>P</i> = 0.665

range of concentrations where they are active (a bell-shaped dose–response curve with a maximum at picomolar doses).

Although some of the inhibitory peptides mentioned here have served as a prototypes for design of peptide mimetics and were considered for clinical trials, the precise mechanism of their action is still unknown (Elgjo *et al.* 1998). It has been shown that acidic oligopeptides, including EPP, can be phosphorylated by protein kinase N II (Bramucci, Miamo & Amici 1992). In phosphorylated form they bind to DNA, competing with transcription factors, and therefore behave as inhibitors of transcription (Chillemi *et al.* 1991; Angiolillo *et al.* 1993). This mechanism could probably not be translated to MTP, since it has a substituted C-terminus and therefore does not fall into a group of acidic peptides. In addition it does not contain serine or other convenient phosphorylation sites as does EPP. Since *in vitro* experiments showed that MTP inhibits transition of melanocytes from the G₂ phase of the cell cycle into mitosis (Gembitsky *et al.* 1998a), it could be suggested that a similar mechanism is taking place *in vivo*. In addition, indirect *in vivo* action of MTP cannot be excluded. Hairless mice (*hr/hr*) have normal immune system in contrast to nude mice (*nu/nu*). MTP may therefore facilitate activation or development of cytotoxic T lymphocytes which could restrict tumour progression (Kawakami *et al.* 1997, 1998). Nevertheless, all hypotheses mentioned here remain speculative and the work is currently in progress to clarify how tumour growth is delayed.

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