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Effect of a mammary-derived growth inhibitor on the expression of the oncogenes c-fos, c-myc and c-ras

W. Lehmann, M. Strauss, U. Kießling, H. Graetz, A. Koberling and P. Langen

Central Institute for Molecular Biology, Academy of Sciences, Robert-Roessle-Str. 10, 1115 Berlin-Buch, GDR

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A mammary-derived growth inhibitor (MDGI) inhibits the resumption of growth of stationary Ehrlich ascites carcinoma (EAC) cells in vitro. The present study shows that the resumption of growth is accompanied by a rapid increase of the steady state mRNA level of the proto-oncogenes c-fos, c-myc and c-ras, which is reduced by MDGI. EAC cells from the exponential growth phase insensitive to MDGI did not show a reduced RNA expression. The effect of MDGI represents a novel activity at the level of gene expression and suggests a link to exist between growth inhibition and the reduction of c-fos, c-myc and c-ras expression.

Oncogene; Growth inhibitor; (Ehrlich ascites cell)

1. INTRODUCTION

Mammary-derived growth inhibitor (MDGI), isolated from bovine mammary glands, is a member of the small family of well defined growth inhibitors [1–3]. It inhibits the resumption of growth of EAC cells, taken from the stationary phase of growth in vivo, in a suspension culture. In contrast, EAC cells from the exponential phase of growth are not inhibited. The dependence of MDGI action on the growth state of the cells and on the presence of counteracting growth factors like EGF or insulin [4–6] suggests its involvement in the balance between positive and negative regulators mediating the regulation of cell proliferation. In the present study we investigated if the growth inhibition is reflected at the level of expression of c-fos, c-myc and c-ras, oncogenes supposed to be involved in growth regulation [7–9].

2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were obtained from the indicated sources: pure mammary-derived growth inhibitor (self-prepared as described previously [3]); *Bgl*II-*Sal*I fragment of v-fos from FBJ murine osteosarcoma virus cloned into *Bam*HI-*Sal*I sites of pAT153 by substitution (kindly supplied by N. Teich, ICRF, England); *Eco*RI-*Eco*RI fragment of genomic DNA containing permuted provirus of avian myelocytomatosis virus, strain MC29, cloned into the *Eco*RI site of pBR313 (kindly supplied by M. Bishop, University of California, USA); *Bgl*I-*Sal*I fragment of Harvey murine sarcoma virus cloned at the *Eco*RI site of pBR322 (kindly supplied by D.R. Lowy, NIH, USA); uridine 5′-[α-^{32}P]triphosphate (spec. act. 800 Ci/mmol) (Radiochemical Centre Amersham, England); nitrocellulose paper (Sartorius, FRG); pGEM plasmids (Promega Biotec, USA); restriction endonucleases (Boehringer Mannheim, FRG).

2.2. *Cell culture*

A hyperdiploid strain of the Ehrlich ascites mammary carcinoma was used. The cells taken freshly from the peritoneal cavity of mice (exponentially growing cells 6 days, stationary cells 12 days after transplantation) were cultivated in suspension according to Negelein et al. [10] as described in detail elsewhere [5]. Stationary cells are not proliferating but fully viable [11,12] and resume growth after transplantation or in culture. After incubation of 15×10^6 cells per sample (10^6 cells/ml medium 199 supplemented with 4% calf serum) for the indicated times, cells were carefully washed with PBS and stored as a pellet in nitrogen up to RNA preparation. Aliquots had previously been transferred to the routine cell culture for measuring the increase in cell number after 24 h. Differences between duplicate samples were below 5% of the mean value.

2.3. *RNA preparation and analysis*

Total cellular RNA was isolated by the guanidinium isothio-

Correspondence address: W. Lehmann, Division of Cellkinetics, Central Institute for Molecular Biology, Academy of Sciences, Robert-Roessle-Str. 10, 1115 Berlin-Buch, GDR

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cyanate method described by Chomczynski and Sacchi [13]. RNA samples were quantified by measurement of A_{280} and the purity was checked by measurement of A_{280}. For RNA slot analysis samples containing 1 or 3 μg RNA were transferred onto a nitrocellulose filter.

The presence of c-fos, c-myc and c-ras RNA was probed using radioactively labelled antisense RNA probes. To this end, a *PstI-SalI* fragment (0.5 kb) of v-fos, a *PstI* fragment (1.5 kb) of v-myc and an *EcoRI* fragment (0.4 kb) of c-ras were subcloned into the multicloning site of pGEM plasmids containing promoters for T7 and SP6 polymerases.

The synthesis of labelled RNA using linearized plasmids was performed according to Melton et al. [14] using about 100 μCi [α-^{32}P]UTP per reaction and 30–60 units of T7-RNA-polymerase resulting in nearly 100% incorporation (162 ng, 1.35×10^9 dpm/μg RNA).

The hybridization and wash procedures were done according to Church and Gilbert [15]. Autoradiography was done at −70°C for 2–5 h using intensifying screens.

3. RESULTS

Fig.1 presents slot blots of two RNA samples prepared in parallel from in vivo cells and illustrates their good agreement. However, we evaluated only differences estimated to be larger than 3-fold. In the experiments we preincubated the cells for 2 h in serum-free medium to accommodate them to culture conditions. We then added serum after 2 h since all earlier experiments on growth inhibition by MDGI were also done with serum-containing medium. In the experiment shown in fig.1 we see a very strong increase of c-fos, c-myc and c-ras gene expression after 15 min and strong inhibition of this induction by MDGI. The fos and ras inductions were already abolished after 60 min whereas the myc induction seemed to be more stable and also partly recovered in MDGI-treated cells. Increase in cell number was 69% for the controls and 43% for the treated cells corresponding to 38% inhibition which is well within the range of inhibition previously reported for MDGI [5,16]. A suppression of the induction was seen in each experiment even if the time course was not always the same. With EAC cells taken from the exponential growth phase in vivo and, therefore, highly proliferating and resistant to MDGI with regard to growth inhibition, MDGI did not suppress oncogene expression. In contrast, MDGI prevented the decrease of mRNA levels of c-fos and c-myc while an induction of ras is even strongly stimulated (fig.2). Increase in cell number

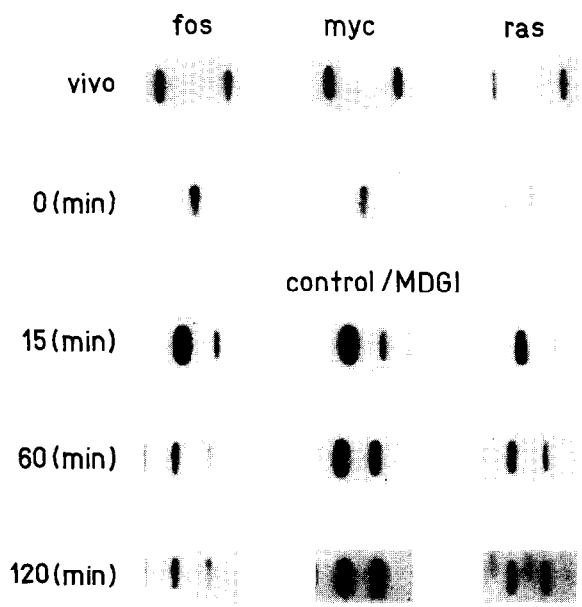

Fig.1. Determination of c-fos, c-myc and c-ras gene expression in stationary EAC cells in vivo (2 parallel samples), after 2 h incubation of these cells in serum-free medium (0 min) and further incubation in serum-containing medium without MDGI (control, left slot) or with MDGI for 15, 60 and 120 min.

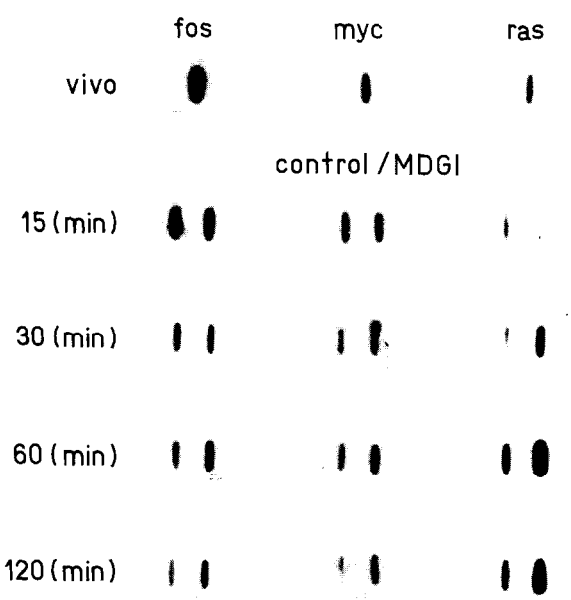

Fig.2. Determination of c-fos, c-myc and c-ras gene expression in EAC cells from the exponential growth state (vivo) and after incubation in serum-containing medium with MDGI (MDGI) or without (control) for 15, 30, 60 and 120 min.

was 76% for controls and 75% for the treated cells.

4. DISCUSSION

All three oncogenes were present already in cells obtained from the stationary phase in vivo. This is in agreement with the demonstration of the presence of c-myc and c-fos in a c-DNA library of EAC cells in vivo [17]. In culture a strong induction took place which is suppressed by MDGI. Though there were differences in the time course of the induction and its suppression, all three oncogenes responded to MDGI. In rapidly proliferating cells, MDGI did not suppress oncogene induction but, in contrast, stabilized or increased it. Since these cells are also resistant to growth inhibition by MDGI, the results suggest a link to exist between growth suppression and oncogene induction.

Compared to stimulating growth factors, much less is known about the existence and role of physiological growth inhibitors with the exception of interferon, tumor growth factor β and tumor necrosis factor, all of which have been described to suppress c-fos, c-myc and/or c-ras expression [18–21]. The present results show that an inhibitor which is neither structurally nor functionally related to these also has strong effects on oncogene expression.

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