Volume 213, number 1, 89–94 FEB 04476 March 1987

Platelet-derived growth factor

Phorbol ester induces the expression of the B-chain but not of the A-chain in HEL cells

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Received 3 December 1986

It was shown previously [(1984) EMBO J. 3, 453–459] that after treatment of the human erythroleukemia cell line HEL with phorbol ester and dimethyl sulfoxide there was a marked increase in the amounts of megakaryotic markers, especially of platelet α-granule proteins and platelet glycoproteins. In order to investigate this differentiation process further we have studied the expression of the mRNA encoding PDGF-A and PDGF-B (c-sis). Upon addition of the phorbol ester to the culture medium the expression of the c-sis transcript was enhanced about 7-fold over a period of 4 days. With dimethyl sulfoxide there was no significant stimulation of the expression. Addition of cycloheximide to HEL cells treated for a short period with phorbol ester superinduced the expression of the c-sis gene. The HEL cells did not express the A-chain mRNA even in the presence of phorbol ester or dimethyl sulfoxide. This leads us to propose that synthesis of the PDGF-A chain and PDGF-B chain is differentially regulated in the megakaryocytic-like HEL cell line.

Megakaryocytic differentiation; Platelet-derived growth factor; Phorbol ester; (HEL cell)

1. INTRODUCTION

The polypeptide platelet-derived growth factor (PDGF) is a potent mitogen for cultured cells of mesenchymal origin (review [1]). The main source of PDGF in human serum is platelets, which are released from megakaryocytes. PDGF is thought to be synthesized in megakaryocytes and then packaged into the α-granules of platelets [2]. Thus platelets appear to be a storage vehicle for PDGF and other mitogens that are released when platelets are exposed to foreign surfaces during blood clotting or when they adhere to sites of blood vessel injury.

PDGF-like proteins are also secreted by cells in culture: the monocytes as circulating cells, endothelial cells and smooth muscle cells as resident cells and in several transformed cell lines like blastoma and sarcoma cell lines and erythroleukemia cells [1,3].

Sequence analysis of human PDGF from platelets after reduction of the disulfide bonds revealed two distinct but related sequences [4], suggesting that PDGF from human platelets is either a homodimer of two equal chains or a heterodimer of two different chains. These chains were subsequently termed A and B [5]. PDGF from porcine

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

platelets appears to consist of B-B homodimers [6] while PDGF secreted by an osteosarcoma cell line is an A-A homodimer [7].

The B-chain of PDGF is highly homologous (greater than 90%) to the predicted amino acid sequence of $p28^{v\text{-}sis}$, the putative transforming protein of simian sarcoma virus (SSV). This led to the suggestion that transformation by SSV might be mediated by production of a PDGF-like molecule through an autocrine stimulation mechanism [4,8].

The human hemopoietic cell line HEL was established from a patient with Hodgkin's disease who later developed erythroleukemia. This cell line is capable of spontaneous and hemin-induced globin synthesis [9]. HEL cells can be induced to differentiate and express megakaryotic markers by treatment with dimethyl sulfoxide or phorbol ester [10]. After DMSO induction 4% of HEL cells show the megakaryocytic phenotype by the appearance of granules with the morphology of platelet α-granules. Before induction with the phorbol ester TPA only 0.01% of the cells were positive for the platelet-specific proteins von Willebrand factor and platelet factor-4. After TPA induction 1% of the cells were positive for this marker and exhibited a marked increase in the characteristic platelet peroxidase activity [10]. HEL cells may therefore provide a suitable system to study differentiation to platelets and the expression and biosynthesis of platelet-specific proteins, investigations which are otherwise hampered by the low abundance of megakaryocytes in the bone marrow [11].

We have used Northern blot analysis to investigate the expression of the PDGF B- and PDGF A-chains in HEL cells during differentiation and could show that with the tumor-promoting agent phorbol 12-myristate 13-acetate (PMA) only the synthesis of the PDGF-B mRNA can be induced. Although the PDGF A-chain is constitutively expressed in different transformed cells and in human placenta, the mRNA for this growth factor cannot be detected in HEL cells using the Northern blot technique.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The HEL cell line was a gift from Dr P. Martin (Seattle, USA). PMA, cycloheximide and A23187 were purchased from Sigma (St. Louis, USA). Cells were grown in RPMI 1640 medium containing 10% fetal calf serum supplemented with 2 mM glutamine, 1 mM pyruvate and antibiotics (100 U penicillin, 100 μg streptomycin and 50 μg gentamycin per ml) in a humidified atmosphere with 5% CO_2. The human osteosarcoma cell line U-20S was kindly provided by Dr B. Westermark (Uppsala, Sweden). The human bladder carcinoma cell line T 24 was from the American Type Culture Collection (ATCC HTB4; Rockville, USA). The human fibrosarcoma cell line HA1781 was kindly provided by Dr S. Aaronson. Cell lines were grown in DMEM medium containing 10% FCS and antibiotics (100 U penicillin, 100 μg streptomycin and 50 μg gentamycin per ml) in a humidified atmosphere with 10% CO_2. Cells were harvested 1, 2 or 4 days after the addition of inducers. Cells treated with DMSO had a low proliferation rate, whereas PMA rendered the cells adherent and stopped proliferation.

2.2. *RNA isolation and Northern analysis*

Cytoplasmic RNA was isolated from cell populations using the guanidinium/cesium chloride method [12]. Polyadenylated RNA was selected by chromatography over oligo(dT)-cellulose columns (Pharmacia, Sweden). RNA was electrophoresed through 1.2% formamide-agarose gels or 1.2% formaldehyde-agarose gels and blotted onto 0.45 μm nitrocellulose filters (Schleicher & Schüll, Dassel, FRG) using the methods in [13].

A 2.0 kbp *Bam*HI restriction fragment from the c-sis clone pMVW-2 [14] was subcloned into SP6 Gemini plasmids (Promega Biotechn, Madsin, USA) in *E. coli* HB101. Clones with inserts in reverse orientation were selected by restriction mapping. ^{32}P-labeled RNA probes for hybridization to the PDGF B-chain were prepared using *Eco*RI-linearized plasmids and hybridization performed as in [15]. To obtain a probe for the PDGF A-chain a sized cDNA library from U-20S cells [14] was screened by standard techniques [16] using a synthetic 22-mer oligomer complementary to the amino-terminus of the PDGF A-chain gene [17]. Two independent clones were isolated, one with a 1.3 kbp insert (pPGF-1) and the other with a 2.75 kbp insert (pPGF-2). Plasmid DNA isolated from the identified clones was digested with restriction enzymes to characterize the inserts. The

PDGF A-chain probe was the ^{32}P-labeled, nick-translated pPGF-1 *Bam*HI fragment. Hybridization was done as described [13]. After hybridization, autoradiography was done at −70°C for 1–7 days using Kodak XAR-5 films and intensifying screens. The intensities of the bands obtained in different blotting experiments were quantified using a laser densitometer (LKB, Sweden), in order to estimate the level of mRNA expression. The sizes of the RNAs were estimated by comparison with the RNAs of tobacco mosaic virus (6395 b) and brome mosaic virus (3234, 2865, 2114, 876 b) separated in the same gel and stained with ethidium bromide.

3. RESULTS

3.1. *Induction of the c-sis gene in HEL cells*

The basal level of c-sis mRNA expression in untreated HEL cells was very low. PMA treatment of HEL cells resulted in increased levels of c-sis mRNA over a period of 4 days (fig.1). After 1 day there was a slight increase (2-fold, not shown), and after 2 and 4 days PMA induced a 5- and 7-fold increase, respectively, in c-sis transcription. The kinetics of c-sis mRNA induction were comparable to those seen in K562 or HL-60 cells after PMA treatment [18,19].

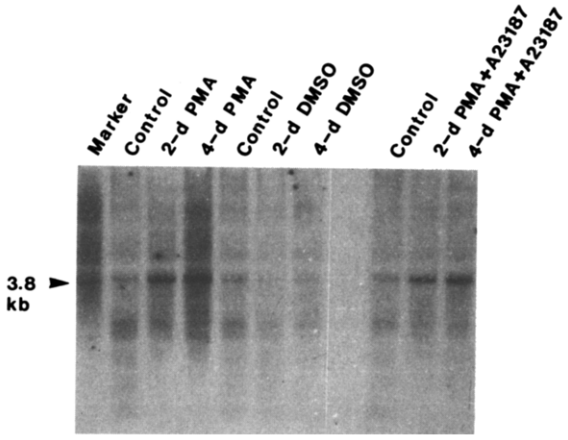

Fig.1. Induction of c-sis mRNA by PMA and DMSO. HEL cell cultures were treated with PMA (160 nM), DMSO (1.25%) or PMA and A23187 (50 nM) for 2 and 4 days, respectively, after which time the cytoplasmic RNA was isolated. RNA aliquots (20 μg) were electrophoresed through a 1.2% formamide-agarose gel. An aliquot (2 μg) of marker cytoplasmic RNA derived from T24 cells was also electrophoresed as an internal control of the hybridization experiment. Hybridization was performed using a c-sis fragment. Autoradiography was performed for 2 days.

To examine the effect of DMSO on expression of the PDGF-B chain, cells were treated for 2 and 4 days, respectively, with DMSO before Northern blot analysis. As shown in fig.1, DMSO had no effect on c-sis mRNA expression in HEL cells. This result was unexpected, since the megakaryocytic phenotype of HEL cells is inducible with both PMA and DMSO.

Synergistic effects have often been observed with phorbol esters and calcium ionophores, e.g. for mitogenesis of lymphocytes [20] and in human blood lymphocyte activation for the expression of the lymphokine interleukin 2 (IL-2) and interferon-gamma (IFN-γ) [21,22]. Moreover, it has recently been shown that the human promyelocytic leukemia line HL-60 differentiated terminally into macrophage-like cells in the presence of subthreshold concentrations of phorbol ester and the Ca^{2+} ionophore A23187 [23].

We investigated whether the simultaneous treatment of HEL cells with PMA and A23187 had a significant effect on the expression of the c-sis mRNA. As shown in fig.2, the cotreatment of the cells did not alter the expression of the differentiation marker.

To determine whether the responses to PMA is a primary induction process HEL cells were treated with PMA together with an inhibitor of protein synthesis, cycloheximide (CH), over a period of 2 h. Addition of CH to PMA-induced cells led to a further increase in c-sis mRNA. After 4 days, expression of the c-sis gene was increased up to 12-fold by this treatment, whereas the expression was increased only 7-fold in cells incubated with PMA alone. These results showed that induction by PMA over a short period in the presence of cycloheximide was independent of de novo protein synthesis.

3.2. *Expression of the PDGF-A mRNA in different cell types*

Because platelets released from megakaryocytes contain both A- and B-chain molecules we also investigated the expression of the A-chain gene in differentiating HEL cells.

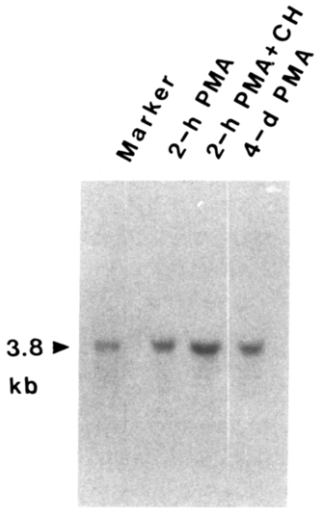

Fig.2. Induction of c-sis mRNA by PMA and cycloheximide. HEL cell cultures were treated with PMA or PMA and cycloheximide (20 μg/ml), respectively, for 2 h and cytoplasmic RNA was isolated after 4 days. RNA aliquots (20 μg) were electrophoresed through a 1.2% formaldehyde-agarose gel. An aliquot (2.5 μg) of marker cytoplasmic RNA derived from T24 cells was also electrophoresed with the RNA samples as an internal control of the hybridization experiment. Hybridization was performed using a c-sis fragment. Autoradiography was performed for 1 day.

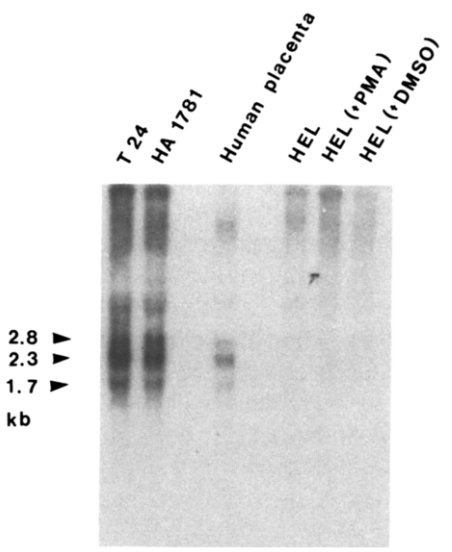

Fig.3. Expression of the PDGF A-chain mRNA in different cell lines. Cytoplasmic RNA was isolated from the transformed cell lines T24 (human bladder carcinoma), HA1781 (human fibrosarcoma), and from human placenta. RNA from HEL cells was isolated after 4 days after the addition of PMA (160 nM), DMSO (1.25%). 10 μg RNA were electrophoresed through 1.2% formaldehyde-agarose gels. Hybridization was performed using a PDGF A-chain cDNA fragment. Autoradiography was performed for 7 days.

As shown in fig.3, uninduced HEL cells and cells after DMSO or PMA induction had no detectable level of PDGF-A mRNA, even when blots were exposed for a prolonged time.

The expression of the A-chain gene was also investigated in two transformed cell lines. The bladder carcinoma cell line T24 is known to secrete PDGF-like activity into the medium. This cell line and also the fibrosarcoma cell line A1781 both expressed c-sis mRNA (Weich, H.A., unpublished). Both cell lines expressed in approximately equal amounts three major transcripts of 2.8, 2.3 and 1.7 kbp. This pattern has also been reported recently [17]. The exact relationship between the different mRNA species for PDGF-A is unknown but is believed to be the result of different splicing processes of the primary transcript.

At this time, from primary cells only macrophages were examined for PDGF A-chain expression. They were negative for A- and B-chain expression [17]. Human placenta was found to be positive for B-chain expression [24]. We have used human placenta RNA to examine the expression of the PDGF A-chain. We found PDGF A-chain mRNA expression to be one-tenth of that detected with the two investigated transformed cell lines.

4. DISCUSSION

It was reported previously that PMA induction of HEL cells led to the synthesis of platelet proteins [10]. Although markers for platelet glycoproteins were detectable before induction, all the platelet markers increased following induction by DMSO and PMA, especially the α-granule proteins von Willebrand factor and platelet factor 4 [10].

In untreated HEL cells the c-sis mRNA expression is about 1/10 of that observed in T24 tumor cells (fig.2). Since T24 cells exhibit a similar level

of expression of c-sis mRNA to that in U-2 OS cells (Weich, H.A., unpublished), which were in turn shown to express about 10–20 copies per cell [25,26], our results indicate an expression rate of 1–2 copies per untreated HEL cell. Accordingly, the observed 7-fold increase in c-sis mRNA expression induced by PMA corresponds to 7–14 copies per cell. On the other hand only 1% of the PMA-induced HEL cells synthesized other α-granule proteins (von Willebrand factor and platelet factor 4) [10]. If this also holds for PDGF, a small percentage of the cells might express about 700–1400 copies of the c-sis gene.

We also showed that treatment with DMSO did not lead to an expression of the c-sis mRNA. These data suggest that the differentiation processes induced by DMSO or PMA respectively, are different, at least in relation to the synthesis of platelet proteins. Similar findings were reported for c-sis gene expression in the human myeloid leukemia cell line HL-60 during monocytic differentiation. In the latter case c-sis mRNA was also detected in PMA-treated cells but not in DMSO-induced cells [19].

The effects of phorbol ester on cells are thought to be mediated by its ability to mimic the action of diacylglycerol in the activation of the protein kinase C, leading to a cellular response or a differentiation pathway [27]. Activation of protein kinase C by diacylglycerol or phorbol ester leads to stimulation of a signal pathway for short-term and long-term cellular responses. The expression of the proto-oncogenes c-fos and c-myc was rapidly stimulated in phorbol ester-treated cells [18,28]. On the other hand, maximum induction of the c-sis mRNA within 4 days in HEL cells, cell lines HL-60 and K 562 [18,19] suggests that the c-sis gene is regulated indirectly by the biochemical events that may follow the activation of the protein kinase C or by independent pathways.

The PDGF A- and B-chains are related in their amino acid sequence and both proteins are present in approximately equal quantities in human platelets. Although both proteins are biologically active and bind at the same cell surface receptor, they are coded for by different genes localized on different chromosomes [7,17].

The expression of the PDGF-A mRNA was investigated in some human tumor cell lines. The expression of this gene was found to be independent of expression of the PDGF-B gene and secretion of a PDGF-like growth factor activity correlated with expression of A- but not B-chain mRNA [17]. The biological significance of the presence of both chains remains unknown.

The presence of PDGF A-chain mRNA species was detected in the human tumor cell lines T24, HA1781 and human placenta using A-chain-specific cDNA probes (fig.3). Hybridization with the same probe under stringent hybridization conditions indicated the absence of PDGF-A mRNA species in HEL cells, not only in untreated cells but also after induction with DMSO or with the phorbol ester PMA.

Thus the present data suggest that expression of the PDGF-B gene and that of PDGF-A are controlled independently of each other in megakaryocytic-like cells.

ACKNOWLEDGEMENTS

We thank Dr P. Martin for providing us with the HEL cells and Dr S. Aaronson for the cell line HA1781. The expert technical assistance of Gabriele Kaszemek is appreciated. We thank Inge Dortmund for typing the manuscript and Dr J.E.G. McCarthy for linguistic advice. H.A.W. is supported by a Fellowship from the Deutsche Forschungsgemeinschaft (HO-996/1-2).

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