Proc. Indian natn. Sci. Acad., **B60**, No. pp, 533-540 (1994)

Induction of Proliferation Specific Proteins by Placental Extracts in Mouse Embryo Fibroblasts

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(Received on 22 February 1993; after revision 19 September 1994; Accepted on 22 September 1994)

Stimulation of quiescent cultures of mouse embryo fibroblasts with placental S-30 (30,000xg supernatant) resulted in the induction of synthesis of a protein of molecular weight 29,000. This protein was shown to be identical to the serum-induced 29kDa protein by peptide mapping. Unlike serum, placental S-30 mediated the induction of this protein in a persistent manner upto 12 hr after addition of the extract. This persistent induction was not due to the lack of progression factors. The placental factor responsible for the induction of the 29kDa protein was found to be a potent heat-stable protein of molecular weight greater than 30,000. Like fetal calf serum (FCS), the placental S-30 also induced the expression of two secreted proteins of molecular weights 48,000 and 26,000. Placental 100,000xg supernatant (S-100) fractions also induced the same set of intracellular proteins as placental S-30. Further, the S-100 fraction induced DNA synthesis when added to quiescent cultures of mouse embryo fibroblasts. These results suggest that placental extract can mimic fetal calf serum in the induction of proliferation specific proteins.

Key Words: Human placenta, Growth factors, DNA synthesis

Introduction

Growth factors play a pivotal role in the regulation of mammalian cell proliferation and a large number of them have been identified (James & Bradshaw 1984). The main function of serum as a tissue culture media supplement is, to supply these growth factors (Pledger et al. 1977). Serum growth factors have been shown to mediate a definite set of events in quiescent cells in the process of stimulating them to undergo the division cycle (Rozengurt 1986). These events include ion fluxes across the plasma membrane, changes in the concentrations of intracellular cyclic nucleotides, phosphorylation of intracellular proteins, transient induction of cellular oncogenes, and regulated expression of other cell cycle specific genes (Shanmugam 1993).

Placenta, which nourishes the fetus throughout its development, has been shown to contain several growth factors. Platelet-derived growth factor (PDGF) (Marez et al. 1987), fibroblast growth factor (FGF) (Gospodarowicz et al. 1985) insulin like growth factor-I (IGF-I) (Mills et al. 1986), and granulocyte macrophage-colony stimulating factor (GM-CSF) (Cukrova & Krkal 1987) have been detected in human placenta, and at least some of them have been partially purified. Based on these studies, we investigated whether placental extract can substitute serum in supporting the growth of cultured cells by look-

ing for the expression of cell cycle specific genes, in terms of their protein products.

Materials and Methods

Cells and Culture

Primary cultures of Swiss mouse embryo fibroblasts were obtained from 13-14 days old embryos. The cultures were maintained in Eagle's minimum essential medium (MEM) containing 10% bovine serum (Sera Lab., U K) and gentamycin (50%g/ml). Cultures from fourth passage onwards (secondary cultures) were used for the experiments. Quiescent cells were obtained by maintaining sub-confluent cultures in medium containing 0.5% serum for 72hr. The cells were released from serum-arrest by the addition of MEM containing 20% FCS or indicated concentrations of placental extract. Platelet poor plasma (PPP) was prepared as described by Pledger et al. (1977).

Preparation of Placental Extract

Healthy human term placentae were transported in ice cold transport buffer (T buffer, 50 mM *Tris*-HCl *p*H 7.6, 25 mM KCl, 100 mM NH_4Cl) and processed within an hour after parturition. The cord and membranes were trimmed off and the placental tissue was cut into small pieces. After washing thoroughly in T buffer, and subsequently in PBS, the tissue pieces were suspended at 1 : 2 ratio (w/v) in PBS and homogenized in a Sorvall Omnimix. The homogenate was centrifuged at 30,000xg for 20 min and the supernatant (S-30) was again centrifuged at 100,000'g for 1hr. Both S-30 and S-100 (100,000xg supernatant) fractions were stored in aliquots at -20$C and were filtered before use through 0.42%M membrane filter after reconstitution with medium.

Radiolabelling of Intracellular and Secreted Proteins

Sub-confluent monolayers (120 cm^2) of serum-starved and stimulated cells were washed with PBS and incubated with 50%Ci of [^{35}S]methionine (Amersham, specific activity 1020Ci/mmol) for 30 min in Hank's balanced saline (HBS). At the end of the labelling period, the monolayers were washed with PBS and incubated in serum-free chase medium for another 30min After chase incubation, the medium was collected and the labelled cells were washed with PBS and lysed in electrophoresis sample buffer (60mM Tris *p*H 6.8, 2% SDS, 4% BME, 0.006% bromophenol blue and 15% glycerol). The chase medium was clarified by centrifugation at 2,000 × g for 10min and the secreted proteins were precipitated with 10% trichloroacetic acid (TCA) in the presence of 50 ug of BSA as carrier. After 30 min incubation at 4$C, the samples were centrifuged at 10,000 × g for 15 min The pellet was dissolved in 0.1 N NaOH and required amounts of solubilized proteins were mixed with equal volumes of 2 ' electrophoresis sample buffer.

Polyacrylamide Gel Electrophoresis and Fluorography

Intracellular and secreted proteins of serum starved and stimulated cells were resolved by electrophoresis in high resolution polyacrylamide gradient (5-18%) gels containing SDS (Laemmli & Favre 1973). Following electrophoresis, the gels were stained and fluorographed using PPO as the scintillant (Bonner & Laskey 1974).

Peptide Mapping

Peptide mapping was done by chemical cleavage, using N- chlorosuccinimide (NCS) as described by Goding and Handman (1984). Briefly, [^{35}S]methionine labeled 29kDa bands were cut from the dried gel, swollen in acetic acid:urea:H_2O (AUW, 1 ml:1g:1ml) and treated with 15 mM NCS in AUW for 20 min The gel pieces were washed and placed in the wells of a 10-18% polyacrylamide gel and electrophoresed. Following electro- phoresis the gels were dried and fluorographed. Quiescent, serum (FCS)

stimulated and placental S-100 treated MEF were incubated for 18hr and then labeled for 30min with 5%Ci/ml of [^{3}H] thymidine (specific activity 18 Ci/m mole). Thymidine incorporation into DNA in terms of TCA precipitable cpm was determined as described earlier (Srinivas et al. 1991).

Results and Discussion

Induction of 29 kDa Protein by Placental Extracts

When serum-starved quiescent cultures of mouse embryo fibroblasts were stimulated to grow by the addition of 20% FCS, a distinct set of proteins were induced (Olashaw & Pledger 1983). Of these, a protein of Mr 29,000 showed marked cell cycle related kinetics of expression (Olashaw & Pledger 1983; Subramaniam & Shanmugam 1986a). When 25% placental extract (S-30) was used in lieu of 20% FCS, essentially, the same set of proteins were induced (figure 1). Densitometric quantitation revealed that the levels of induction of two proteins of Mr 29,000 and 42,000 by 20% FCS (lane D) or 25% placental S-30 (lanes B and C) were the same. Specifically, the induction of the 29kDa protein was marked in both serum-induced and placental extract treated cells whereas quiescent cells expressed negligible quantities of this protein. The N-chlorosuccinimide cleavage pattern of placental S-30 induced 29 kDa protein (data not shown) was identical to that of the serum induced 29 kDa protein reported earlier (Subramaniam & Shanmugam 1986a).

Persistent Induction of 29kDa Protein by Placental S-30

When quiescent cultures of mouse embryo fibroblasts were stimulated with placental S-30 for variable lengths of time, the 29 kDa protein induction was persistent upto 12 hr after stimulation (figure 2). Densitometric quantitation showed that the level of induction of this protein was more than two fold compared to its level in the quiescent cells throughout this period. The 29 kDa protein was previously shown to be a nuclear protein strongly associated with chromatin (Subramaniam & Shanmugam 1986b, Olashaw & Pledger 1983). Pledger et al. (1981)

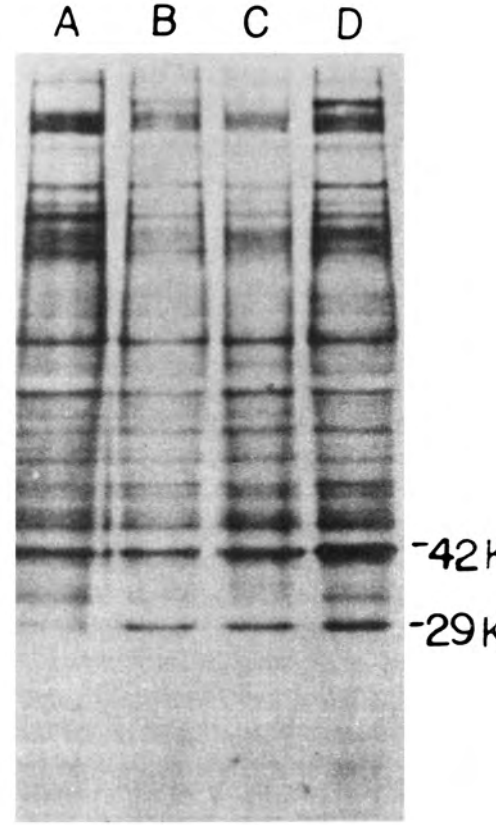

Figure 1 Comparison of induction of intracellular proteins in quiescent cells treated with fetal calf serum and placental extracts (S-30). Quiescent cultures of MEF were treated with 20% FCS or 25% placental S-30 for 3 hr. Cells were labelled with [^{35}S]-methionine for 30 min in serum free medium. Following a 30 min chase incubation, cells were lysed in electrophoresis sample buffer. The labelled proteins were analyzed by electrophoresis in 5-18% polyacrylamide gradient gels containing SDS and fluorographed. *Lane A*, quiescent cells; *lanes B and C*, cells treated with 25% placental S-30 prepared in PBS and MEM, respectively; *lane D*, cells stimulated with 20% FCS

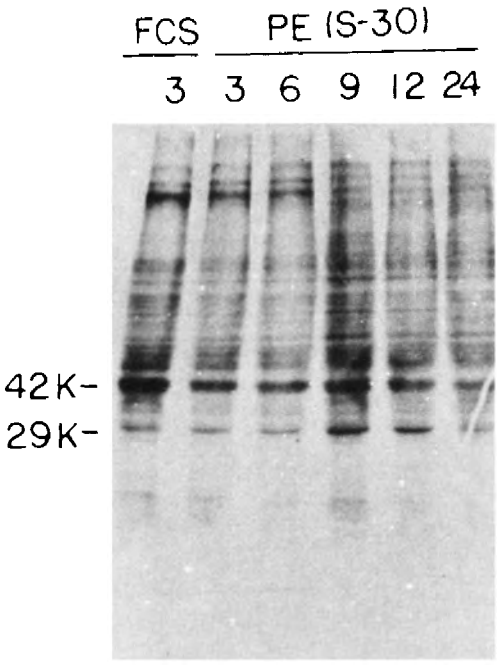

Figure 2 Persistent induction of 29 kDa protein synthesis in quiescent cells treated with placental (S-30). Quiescent cultures of MEF at different time points after treatment with 25% placental S-30 were labelled with [^{35}S]-methionine for 30 min. Labelled intracellular proteins were electrophoresed in 5-18% SDS-polyacrylamide gradient gels and fluorographed, The numbers above each lane indicate the time (in hours) of radiolabelling after the addition of FCS or placental S-30

suggested that this protein could be a competence protein, as it was induced by serum and other competence factors such as PDGF and FGF but not by progression factors such as EGF and insulin. Serum factors induced the 29kDa protein transiently in the first 2-3 hr of stimulation and its level of expression declined to undetectable levels in about 6 hr after mitogenic stimuli (Subramaniam & Shanmugam 1986a).

In order to verify the possibility that the persistent induction of the 29kDa protein by placental S-30 could be due to the absence of progression factors in the placental extract, quiescent cells were stimulated with placental S-30 in the presence of platelet poor plasma (PPP) or with 20% FCS which contained progression factors. In the case of placental S-30 and PPP combination, the 29 kDa protein induction was persistent even after 8 hr of co-stimulation (data not shown). Also with the combination of placental extract and FCS, the cells showed persistent expression of 29kDa protein in the 24hr period tested (figure 3). These results show that the persistent induction of the 29kDa protein

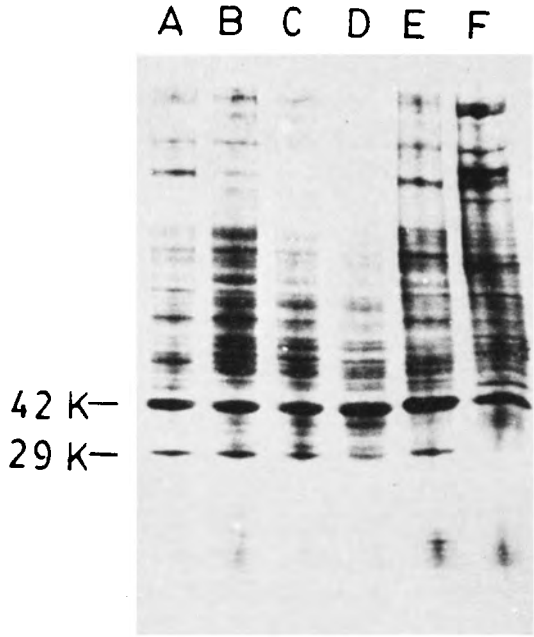

Figure 3 Effect of FCS on placental extract (S-30) mediated induction of the 29 kDa protein. Quiescent cultures of MEF were stimulated with 25% placental S-30 together with 20% FCS for various lengths of time and labelled with [^{35}S]-methionine for 30 min. Following a 30 min chase incubation, cells were lysed in electrophoresis sample buffer. The labelled proteins were analyzed by electrophoresis in 5-18% polyacrylamide gradient gels containing SDS and fluorographed. *Lanes A,B,C and D* show protein profiles of cells stimulated with 25% placental extract + 20% FCS for 3, 9, 15 and 24 hr respectively; *lane E*, protein profile of cells stimulated with 20% FCS alone for 3 hr *lane F*, protein profile of quiescent cells.

by placental S-30 was not due to the lack of progression factors in it. It is not known whether the placental extract contains a factor distinct from known competence factors that mediates the persistent induction of the 29kDa protein or contains a factor which could repress the expression of 29kDa protein directly, or indirectly by altering the expression of some other gene(s) whose product(s) may be involved in the regulation of 29 kDa protein expression.

Characterization of the 29kDa protein inducing factor

The putative factor in placental extracts which induces the 29kDa protein in quiescent cells was shown to be as potent as FCS in the induction of 29kDa protein (figure 4). MEM supplemented with placental S-30 equivalent to 2.5 - 12.5%g/ml of proteins (*lanes A-E*) showed almost the same levels of 29kDa protein induction as that done by 20% FCS having a protein concentration of 9 mg/ml (*lane F*). The factor appears to be present in the soluble fraction of the placental extract (10,000.g supernatant) as both the S-30 and S-100 fractions showed similar activity (figure 5). The factor was not dialysable since placental S-100 dialyzed against PBS did not show any reduction in its ability to induce the

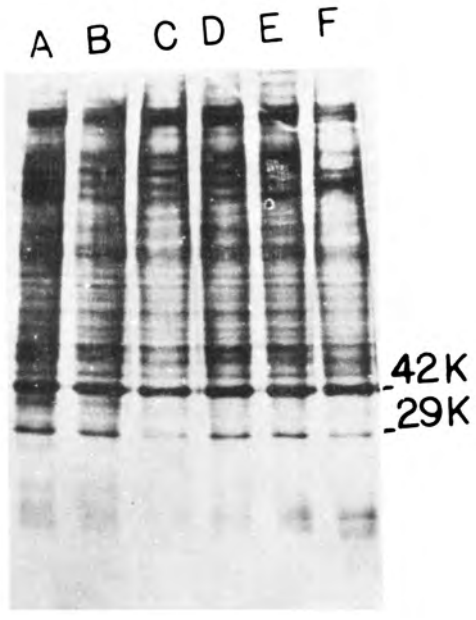

Figure 4 Effect of placental extract (S-30) concentration on the induction of 29kDa protein. Quiescent cells were treated with varying concentrations of placental S-30 for 3 hr. Following 30 min labelling and a 30 min chase, the cells were washed and lysed. The lysates were electrophoresed in 5-18% SDS- PAGE and fluorographed. *Lanes A-E* show the intracellular proteins of cells treated with MEM containing 125, 62.5, 31.25, 7.5 and 2.5 mg per ml of placental S-30 proteins, respectively; *lane F* shows intracellular proteins of cells stimulated with 20% FCS (final protein concentration 9 mg/ml)

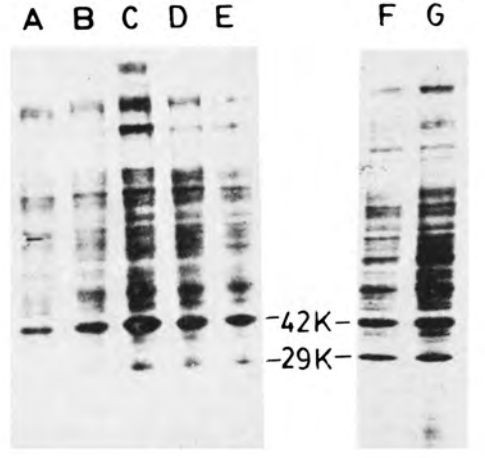

Figure 5 Effect of dialyzed and ultra-filtered placental S-100 preparations on protein synthesis in quiescent cells. Placental S-100 was extensively dialyzed against PBS for 48 hr with two changes or concentrated by ultra filtration. Quiescent cultures were treated with these extracts for 3 hr and labelled with [^{35}S]- methionine for 30 min. Following a 30 min chase incubation the intracellular proteins were fluorographed. *Lane A*, intracellular proteins of quiescent cells; *lanes B, C, D* and *E* show profiles of intracellular proteins of cells treated with 0.5% serum, 20% FCS, non-dialyzed placental S-100 and dialyzed placental S-100, respectively; *lanes F and G* contained intracellular proteins of cells treated with placental S-00 concentrated through ultra filters with molecular weight cut off points of 10,000 and 30,000, respectively

29kDa protein (figure 6). Concentrates of placental S-100 obtained by using ultrafilters with molecular weight cut-off points 10,000 and 30,000 retained the activity (figure 6) indicating that the 29kDa inducing factor could be a protein or a complex of proteins of molecular weight greater than 30,000. The factor was not destroyed by heating the medium containing placental S-100 at 100$C for 10 min (figure 6) suggesting that it is a heat stable protein.

Effect of placental extracts on secreted proteins

Figure 7 shows the pattern of secreted proteins of quiescent cells that were stimulated with 20% FCS or with placental S-30. Both induced the secretion of two proteins of Mr the 48,000 and 26,000. In addition, another protein of Mr 60,000 was induced by both FCS and placental S-30 stimulated cells. The 45kDa protein secreted by quiescent, serum and placental S-30 stimulated

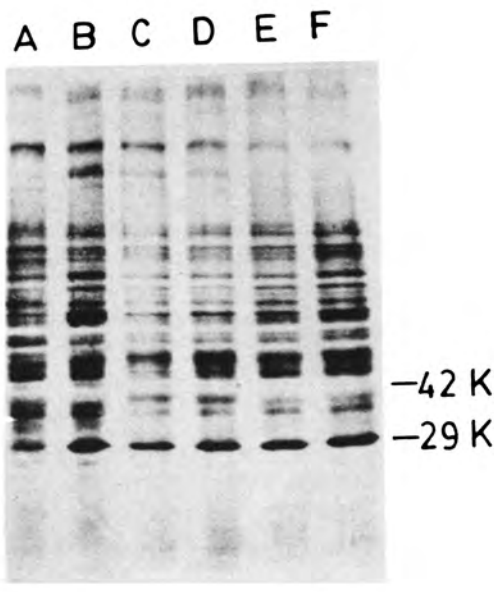

Figure 6 Placental factors that induce the 29kDa protein are heat-stable. MEM containing placental S-30 was subjected to heat treatment before being used for stimulating quiescent cultures of MEF. Treated cells were labelled with [^{35}S]-methionine for 30 min, chased for further 30 min and the protein profiles were analyzed. Lane A, protein profiles of quiescent cells; lane B, protein profiles of cells treated with placental S-30. Lanes C, D and E show protein profiles of cells grown in the presence of placental S-30 preparations treated at 65$C for 15 min (C), 30 min (D) and 45 min (E). Lane F shows the proteins of cells treated with heated (100$C, 10 min) placental S-30.

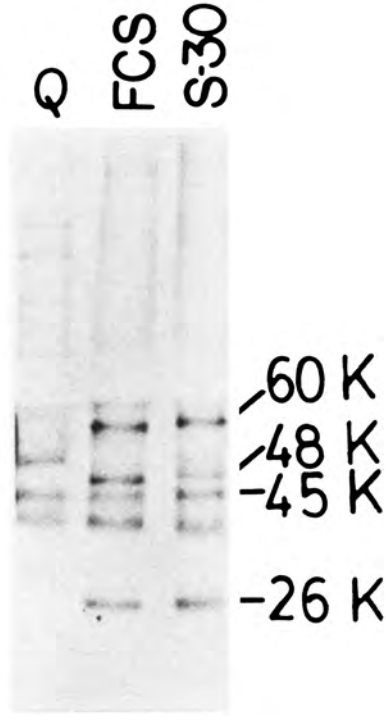

Figure 7 Comparison of secreted proteins of mouse embryo fibroblasts treated with FCS and placental extracts. Quiescent cells were treated with 20% FCS or 25% placental S-30 for 3hr. Following 30 min labelling with [^{35}S]-methionine and a 30 min chase in serum-free medium, the labelled proteins in the chase medium were precipitated with TCA. Precipitated proteins were electrophoresed in 5-18% polyacrylamide gels containing SDS and fluorographed. Secreted proteins of quiescent cells (Q) and cells treated with 20% FCS or 25% placental S-30 are shown.

cells was shown to be absent in S-phase cells and is believed to be an autocrine growth regulator (Nagashunmugam & Shanmugam 1987). The 48kDa protein has been recently identified as plasminogen activator inhibitor (Srinivas, Nagashunmugam & Shanmugam 1990).

Since both S-30 and S-100 fractions of human placenta induced the synthesis of mitogen-induced proteins, the mitogenic potential of S-100 in terms of induction of DNA synthesis was monitored. For these studies, quiescent cultures of mouse embryo fibroblasts were exposed to fetal calf serum (FCS) or placental S-100 and [^{3}H]thymidine incorporation into DNA of these cultures was determined. The results shown in table 1 indicate that placental S-100 is highly mitogenic at a concentration of 150 ug/ml of protein, where a 2.4 fold increase in DNA synthesis was observed compared to the quiescent cells. The values of [^{3}H]thymidine incorporation (shown in table 1) were obtained from TCA insoluble radioactivity and were the mean of duplicate experiments. Although this effect of S-100 is appreciable, it was lower than that observed with FCS. Higher concentrations (300 %g/ml) of S-100 inhibited DNA synthesis and this may be due to an increase in toxic/inhibitory factors.

It has been suggested that specific intracellular and secreted proteins induced by serum could serve as useful markers for monitoring the transition of cells from quiescence to proliferation

Table 1 *Stimulation of DNA synthesis in quiescent MEF by placental S-100*

Additions	[^{3}H]-Thymidine incorporation into DNA (cpm/2 X 10^6 cells)	Fold induction
None	900	-
FCS (20%)	4000	4.4
Placental S-100 (150 µg/ml)	2200	2.4

(Subramaniam & Shanmugam 1985). Placental extracts induce the expression of cell cycle specific proteins much the same way as serum. However, the placental extract-mediated induction of the 29kDa competence protein is remarkably persistent for more than 12 hr after stimulation. This and other physical properties of the placental factor(s), which mediate the expression of the gene encoding the29 kDa protein, suggest that placental extract may contain factor(s) other than the already identified growth factors. These factor(s) could mediate the persistent induction of the 29kDa protein either by direct activation of the gene coding for it or by perturbing the expression of genes encoding other cell cycle controlled regulatory proteins.

Acknowledgements

This investigation was supported by M K U Biologicals and the Department of Science and Technology, New Delhi.

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