Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle

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Summary

In vitro produced, 2-cell bovine embryos were cultured in serum-free medium supplemented with various combinations of growth factors to test the hypothesis that these polypeptide factors are able to signal preimplantation development. The developmental arrest that occurs during the 8-cell stage with typical culture methods might be relieved by a growth factor-dependent mechanism that would stimulate expression of the embryonic genome, thereby mimicking events that occur in vivo in the oviduct during the fourth cell cycle (8- to 16-cell stage). Subsequently, other growth factors might promote compaction and blastulation, processes which normally occur in the uterus.

The effects of growth factors on early embryos were evaluated using phase contrast microscopy to monitor progression to the 8-cell stage, completion and duration of the fourth cell cycle, and blastocyst formation.

Platelet derived growth factor (PDGF) promoted development beyond the 16-cell stage in 39.1% of the 2-cell embryos examined in all experiments. The duration of the fourth cell cycle among these embryos was approximately 26 hours. During development after the 16-cell stage, PDGF reduced the proportion of embryos blastulating from 12.7% to 5.8%; in contrast, transforming growth factor α (TGFα), acting during the same developmental time period, increased the proportion of embryos blastulating from 8.6% to 40.6%.

These results, using serum-free medium, indicated that PDGF signalled completion of the fourth cell cycle. TGFα, and perhaps basic fibroblast growth factor (bFGF), promoted blastulation of 16-cell embryos during subsequent culture.

Key words: platelet derived growth factor (PDGF), bovine embryos, maternal-embryonic transition.

Introduction

In vitro cultured bovine embryos commonly arrest development during the transition from a regulatory program governed by proteins transcribed from maternally synthesized mRNAs stored in the oocyte to a regulatory program controlled by proteins synthesized from the embryonic genome (Barnes and Eyestone, 1990). This transition occurs primarily during the fourth cell cycle as the embryo grows from 8 to 16 cells. It is accompanied by major changes in profiles of protein synthesis (Frei et al., 1989), new synthesis of ribosomal RNAs (Kopecny et al., 1989), and transcriptional dependency (Barnes, 1988). Culture in typical media inhibits completion of this transition as evidenced by a developmental block that occurs during the 8-cell stage (Thibault, 1966; Camous et al., 1984).

Other cells cultured in vitro also enter a period of arrest or dormancy with respect to growth, a state termed G0 (reviewed by Baserga, 1985). Similar to certain cells in G0, developmentally arrested bovine blastomeres are smaller than their parent cells (Hamilton and Laing, 1946), have declining rates of protein synthesis (Frei et al., 1989), can be mitotically stimulated by human (but not fetal bovine) serum (McLaughlin et al., 1990), and, if analogous to murine embryos, have increased rates of mRNA turnover (Brinster et al., 1980, Píkó and Clegg, 1982).

Relief from G0 arrest depends on several factors, including PDGF and insulin-like growth factor I (IGF-I) (reviewed by Campisi, 1989). Growth arrested 3T3 cells stimulated with PDGF express the cell cycle specific proto-oncogenes myc and fos (Müller et al., 1984, Burck et al., 1988) and enter mitosis within 22-24 hours (Pardee, 1989). Subsequent to PDGF, IGF-I is required by growth arrested 3T3 cells for initiation of DNA synthesis (Campisi et al., 1984), and expression of the GTP-binding proto-oncoprotein ras (Lu et al., 1989).

Upon completion of the transition from maternal to embryonic control of development, epidermal growth factor (EGF) enhances compaction and blastulation in murine embryos (Wood and Kaye, 1989). Transforming growth factor α, which is very similar in activity to EGF (Bascom et al., 1989) and may be a fetal form of EGF (Mercola and...
Stiles, 1988), is secreted by uterine tissue (Huet-Hudson et al., 1990). Basic fibroblast growth factor (bFGF), itself a potent mitogen (Burgess and Maciag, 1989), is also present in uterine secretions (Brigstock et al., 1989). We have previously demonstrated that bFGF, in combination with transforming growth factor β (TGFβ), relieves developmental arrest and promotes blastocyst formation in bovine embryos (Larson, unpublished data).

The present experiments were designed to examine the effects of PDGF and IGF-I on the growth of bovine embryos cultured in vitro during the transition from maternal to embryonic control of development. Further, the role of TGFα and bFGF in promoting development after the completion of this transition was investigated.

**Materials and methods**

**Embryo culture**

Cumulus-free bovine embryos were obtained as previously described using abattoir-derived oocytes fertilized with heparin-treated sperm (Larson et al., 1991). Cumulus-free embryos were randomly allocated to treatments in groups of 5 and cultured in 500 µl of medium. All incubations were performed at 39°C in a humidified atmosphere of 5% CO₂ in air using a basal medium of cholesterol-free B2 (Menezo et al., 1984) adjusted to 2 mM calcium and 3 mM glucose.

**Source of growth factors**

Recombinant hPDGF-BB and recombinant bFGF were purchased from Amgen (Thousand Oaks, CA). TGFβ-1, derived from porcine platelets, was purchased from R&D Systems (Minneapolis, MN). Recombinant IGF-I, produced by Pittman-Moore, was provided by Dr. D. H. Beermann. TGFα was purified from medium conditioned by Fischer rat embryo cells, transformed with Syndy-Theilen feline sarcoma virus and was donated by Dr. R. A. Ignatz (Ignatz et al., 1986).

**Experimental design**

These experiments were designed as randomized complete blocks with each block consisting of one group of oocytes harvested, matured, fertilized and cultured together. Unless indicated otherwise, each observation was the proportion of embryos that developed to a specific stage divided by the total number of cleaved embryos within each experimental unit of five embryos. Embryos that did not cleave at least once were not included in the analysis in order to eliminate oocytes that were not competent to mature or to be fertilized.

**Experiment 1**

The purpose of Experiment 1 was assess the similarity of the transition from maternal to embryonic control of development to what is known of the transition from G₀ to G₁ in growth arrested fibroblasts. Specifically, the studies examined the effects of PDGF and IGF-I in combination with the previously documented effects of TGFβ and bFGF. Treatments were arranged in a 2 × 2 factorial design with the main factors being addition to the medium of PDGF (1 ng ml⁻¹) and IGF-I (20 ng ml⁻²). The basal medium (see above) was supplemented with both TGFβ (1 ng ml⁻¹) and bFGF (50 pg ml⁻¹). Treatment was initiated 24 hours after the start of sperm-egg co-incubation and continued for 9 days. Embryos were observed daily with phase contrast microscopy for initial cleavage, progression to the 8-cell stage, development beyond the fourth cell cycle, and morula and blastocyst formation.

There were two blocks of embryos in Experiment 1 with a total of 47 experimental units (235 oocytes, 193 cleaved embryos). Treatment comparisons were made by using analysis of variance with single degree of freedom orthogonal contrasts.

**Experiment 2**

The second experiment determined whether the effect of PDGF was independent of TGFβ and bFGF potentiation. Cumulus-free 2-cell embryos were allocated randomly to treatment with either PDGF alone (1 ng ml⁻¹) or in combination with TGFβ (1 ng ml⁻¹) and bFGF (50 pg ml⁻¹). The treatment began after cumulus cell removal (24 hours after the start of sperm-egg co-incubation) and continued uninterrupted for 9 days. Observations were made as in the previous experiment. Experiment 2 involved two blocks of embryos with a total of 55 experimental units (275 oocytes, 215 2-cell embryos). Comparisons were made using analysis of variance.

**Experiment 3**

Experiment 3 investigated differences in the length of the fourth cell cycle in embryos treated with either PDGF alone or the combination of TGFβ and bFGF. Cumulus-free embryos, as above, were allocated to treatment with either PDGF (1 ng ml⁻¹) or TGFβ (1 ng ml⁻¹) plus bFGF (50 pg ml⁻¹). Observations were recorded every 6 hours beginning 42 hours after the start of sperm-egg co-culture in order to determine the start and end of the fourth cell cycle. Each embryo that developed to the 16-cell stage was considered to be a single experimental unit (n = 25).

**Experiment 4**

Experiments 1 and 2 demonstrated that PDGF alone was as effective as of TGFβ plus bFGF in promoting completion of the fourth cell cycle, but PDGF subsequently appeared to suppress blastocyst formation. Experiment 4 was therefore designed to study the effects of TGFα and bFGF on blastulation of embryos that developed to the 16-cell stage in response to PDGF.

At 56 hours after the start of sperm-egg co-incubation in basal medium alone, 8-cell, cumulus-free embryos were transferred to medium containing PDGF (1 ng ml⁻¹). After incubation for 24 hours (80 hours after fertilization), 8- to 16-cell embryos were washed in PDGF-free medium and allocated randomly to treatments arranged in a 2 × 2 factorial design, the main factors being additions to the medium of bFGF (50 pg ml⁻¹) and TGFα (1 ng ml⁻¹). Embryos were observed daily for completion of the fourth cell cycle, compaction and blastocyst formation.

This experiment was a randomized complete block design with two blocks of embryos and 36 experimental units (178 8- to 16-cell embryos). Observations were tabulated as the number of embryos that blastulated divided by the number that completed the fourth cell cycle.

**Results**

**Experiment 1**

There was no observed effect of PDGF or IGF-I during the first three cell cycles. The treatment means for the proportion of 2-cell embryos developing to the 8-cell stage were 54.5%, 66.0%, 60.1% and 50.8% for the control, IGF-I, PDGF and IGF-I plus PDGF-treated embryos, respectively.
There was also no effect of either PDGF or IGF-I on the proportion of embryos completing the fourth cell cycle (Table 1). The main effect of PDGF was a slight reduction \( (P < 0.65) \) in the proportion of embryos completing the fourth cell cycle from 46.3% to 41.2%. 48.1% of the embryos treated with IGF-I completed the fourth cell cycle as compared to 39.2% of the embryos that were not exposed to IGF-I \( (P < 0.31) \).

Subsequent to the completion of the fourth cell cycle, PDGF decreased the proportion of embryos blastulating from 12.7% to 5.8% \( (P < 0.057) \) (Table 1). IGF-I was not observed to affect blastulation \( (P < 0.41) \), with 10.0% of the IGF-I treated embryos having blastulated as compared to 8.4% of the embryos not treated with IGF-I.

The rate of development to the 16-cell stage appeared to be accelerated in the embryos treated with PDGF, and Experiment 3 was designed to test this possibility. Embryos began to blastulate 6 and 7 days after the initiation of sperm-egg coculture.

### Experiment 2

A similar proportion of 2-cell embryos treated with PDGF alone \( (39.0 \pm 6.5 \%) \) (mean ± standard error of the mean) among 28 observations totalling 98 embryos) or in combination with TGFβ and bFGF \( (29.1 \pm 6.6 \%) \) among 27 observations totalling 103 embryos) completed the fourth cell cycle \( (P < 0.17) \). The proportion of 2-cell embryos developing to the blastocyst stage was also similar between treatments \( (P < 0.85) \) and averaged 6.8% for embryos treated with PDGF alone and 6.1% for embryos treated with all three growth factors.

### Experiment 3

The length of the fourth cell cycle in PDGF-treated embryos \( (26.0 \pm 2.1 \text{ hours}) \) was only 60% \( (P < 0.001) \) that of embryos treated with TGFβ and bFGF \( (43.7 \pm 2.1 \text{ hours}) \). The proportion of embryos developing to the 16-cell stage did not differ \( (P < 0.65) \) between treatments and averaged 44.8% \( (13/29) \) for the TGFβ and bFGF treated embryos.

Additionally, indirect immunofluorescence studies indicated that PDGF triggered the expression of the proto-oncoproteins myc and fos (data not shown), but confirmation of these results using immunoprecipitation was not attempted because of the limited number of embryos available for labelling.

### Table 1. The mean proportion and standard error of the mean of 2-cell, in vitro produced embryos developing beyond the 16-cell stage and blastulating among groups of embryos cultured in the presence of the growth factors IGF-I and PDGF

<table>
<thead>
<tr>
<th>PDGF</th>
<th>0.0 ng ml(^{-1})</th>
<th>1.0 ng ml(^{-1})</th>
<th>IGF-I main effect means*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I 0.0 ng ml(^{-1})</td>
<td>Mean proportion developing beyond the 16-cell stage=34.2%±9.4%</td>
<td>Mean proportion developing beyond the 16-cell stage=43.7%±9.0%</td>
<td>Mean proportion developing beyond the 16-cell stage=39.2%±6.5%</td>
</tr>
<tr>
<td></td>
<td>Mean proportion blastulating =13.0±5.0%</td>
<td>Mean proportion blastulating =4.11±4.8%</td>
<td>Mean proportion blastulating =8.4%±3.5%</td>
</tr>
<tr>
<td>Groups (n)=11</td>
<td>Groups (n)=12</td>
<td>Groups (n)=23</td>
<td>Groups (n)=89</td>
</tr>
<tr>
<td>Total embryos=43</td>
<td>Total embryos=46</td>
<td>Total embryos=89</td>
<td></td>
</tr>
<tr>
<td>20.0 ng ml(^{-1})</td>
<td>Mean proportion developing beyond the 16-cell stage=57.4%±9.0%</td>
<td>Mean proportion developing beyond the 16-cell stage=38.7%±9.0%</td>
<td>Mean proportion developing beyond the 16-cell stage=48.1%±6.4%</td>
</tr>
<tr>
<td></td>
<td>Mean proportion blastulating =17.9%±4.8%</td>
<td>Mean proportion blastulating =7.5%±4.8%</td>
<td>Mean proportion blastulating =10.0%±3.4%</td>
</tr>
<tr>
<td>Groups (n)=12</td>
<td>Groups (n)=12</td>
<td>Groups (n)=17</td>
<td>Groups (n)=63</td>
</tr>
<tr>
<td>Total embryos=47</td>
<td>Total embryos=57</td>
<td>Total embryos=63</td>
<td></td>
</tr>
<tr>
<td>TGFα main effect means†</td>
<td>Mean proportion developing beyond the 16-cell stage=46.3%±6.5%</td>
<td>Mean proportion developing beyond the 16-cell stage=41.2%±6.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean proportion blastulating =12.7%±3.5%</td>
<td>Mean proportion blastulating =5.8%±3.4%</td>
<td></td>
</tr>
<tr>
<td>Groups (n)=23</td>
<td>Groups (n)=24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total embryos=90</td>
<td>Total embryos=103</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The basal medium used in all treatments contained both TGFβ \( (1 \text{ ng ml}^{-1}) \) and bFGF \( (0.05 \text{ ng ml}^{-1}) \). Treatment was initiated at the 2-cell stage and continued for 9 days.

There was no interaction between the effects of the two growth factors on either development beyond the 16-cell stage \( (P<0.11) \) or on blastocyst formation \( (P<0.87) \).

*There was no detectable mean effect of IGF-I on either development beyond the 16-cell stage \( (P<0.30) \) or on blastocyst formation \( (P<0.41) \).

†While there was no effect of PDGF on development beyond the 16-cell stage \( (P<0.65) \), the main effect of PDGF on blastocyst formation was to reduce the proportion blastulating from 12.7% to 5.8% \( (P<0.06) \).
Discussion

The composite results of these experiments are consistent with the hypothesis that PDGF initiates gene expression of in vitro cultured bovine embryos during the fourth cell cycle. TGFα subsequently signals continued growth and development of 16-cell embryos and promotes blastulation.

In the present experiment no effect of IGF-I was detected during the transition from maternal to embryonic control of development. Fibroblasts, which require IGF-I to initiate DNA synthesis, arrest cell cycle progression before S phase of the cell cycle but murine embryos, the only species documented, become mitotically arrested after completion of DNA synthesis (Bolton et al., 1984). The IGF-I receptor cannot be detected in murine embryos until the time of compaction (Heyner et al., 1989). These findings and the present results might suggest that the simple embryonic cell cycle lacks some of the checkpoints and controls that are present in normal somatic cells (Murray, 1991).

Proliferation of cultured, growth-arrested bovine embryos, shown here to be induced by PDGF, may be analogous to the growth factor’s effect on other cultured cells (Pledger et al., 1977, 1978), perhaps involving a mechanism that includes synthesis of the regulatory subunit of Mitosis Promoting Factor (Jaskulski et al., 1988). The time between addition of PDGF and completion of the fourth cell cycle was approximately 22-24 hours observed for growth-arrested fibroblasts (Pardee, 1989). First and Barnes (1989) found the length of the fourth cell cycle in their cultured bovine embryos to be 24 hours.

We have previously shown that TGFβ plus bFGF triggered development in growth-arrested embryos (Larson et al., 1991). This action, as in growth arrested cells, most likely involves autocrine synthesis of sis (Leof et al., 1986), a homodimeric isoform of PDGF (Hannink and Donoghue, 1989). Because of the time necessary for synthesis and accumulation of sis (Leof et al., 1986), growth arrested fibroblasts require 12 hours longer to enter mitosis if treated with TGFβ rather than PDGF. In the present experiment, the completion of the fourth cell cycle required 18 hours longer for embryos

### Table 1. Proportion (%±standard error of the mean) of 16-cell bovine embryos developing to the blastocyst stage when treated with the growth factors TGFα (1.0 ng ml⁻¹) and bFGF (0.05 ng ml⁻¹) applied in a factorial arrangement

<table>
<thead>
<tr>
<th></th>
<th>TGFα 0.0 ng ml⁻¹</th>
<th>TGFα 1.0 ng ml⁻¹</th>
<th>bFGF main effect means†</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF 0.0 ng ml⁻¹</td>
<td>Mean among groups=</td>
<td>Mean among groups=</td>
<td>Mean among groups=</td>
</tr>
<tr>
<td>Groups (n)=12</td>
<td>6.3%±9.0%</td>
<td>23.8%±11.8%</td>
<td>12.5%±7.1%</td>
</tr>
<tr>
<td>Total embryos=43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bFGF 0.05 ng ml⁻¹</td>
<td>Mean among groups=</td>
<td>Mean among groups=</td>
<td>Mean among groups=</td>
</tr>
<tr>
<td>Groups (n)=5</td>
<td>13.3%±14.0%</td>
<td>47.9%±9.0%</td>
<td>39.7%±7.6%</td>
</tr>
<tr>
<td>Total embryos=15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFα main effect means†</td>
<td>Mean among groups=</td>
<td>Mean among groups=</td>
<td>Mean among groups=</td>
</tr>
<tr>
<td>Groups (n)=17</td>
<td>8.6%±7.6%</td>
<td>40.6%±7.1%</td>
<td>26.2%±11.5%</td>
</tr>
<tr>
<td>Total embryos=58</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Twenty-four hours before the start of treatment, 8-cell embryos were selected and then incubated in the presence of PDGF.

There was no positive interaction between the growth factors on the proportion of 16-cell embryos forming blastocysts. The estimated interaction was 9.3%±9.0 (P<0.17).

* The main effect of bFGF was to increase significantly the proportion of embryos blastulating by 15.4%±11.5% (P<0.41). Weighted means were used to make this comparison because of the uneven sample sizes.

† The main effect of TGFα was to increase the proportion of embryos blastulating by 26.2%±11.5% (P<0.025). Estimates were made using weighted means.

**Experiment 4**

In this experiment, 76.3% of the harvested oocytes completed the first cleavage division and 43.2% of the cleaved embryos developed to the 8-cell stage in the basal medium without supplemental growth factors. PDGF treatment, for just 24 hours, resulted in 57.0% of these 8-cell embryos completing the fourth cell cycle, and TGFα plus bFGF promoted blastulation in 47.9% of the 16-cell embryos. Following exposure to PDGF, the main effect of TGFα was to increase the number of 16-cell embryos blastulating from an average of 8.6% to an average of 40.6% (P<0.025, Table 2). While statistically insignificant (P>0.41), bFGF increased the number of embryos forming blastocysts from 12.5% to 39.7%. Had the actions of the two growth factors been simply additive, the expected value of the proportion of embryos blastulating in response to both growth factors would have been 30.9%. The observed value was 47.9% (Table 2), yet statistical analysis failed to verify any significant additive or synergistic effects of the growth factors (P>0.41).

Blastulation of the embryos in this experiment began 2 and 3 days after the start of treatment with TGFα and bFGF; that is, on the fifth and sixth day after fertilization.
stimulated with TGFβ plus bFGF than for those stimulated with PDGF.

Human serum has been found to support early bovine embryo development much more efficiently than fetal bovine serum (McLaughlin et al., 1990, Camous et al., 1984). The concentration of PDGF is ten times greater in human serum than fetal bovine serum (Kumar et al., 1988).

It was apparent from the present study that prolonged exposure to PDGF decreased the proportion of embryos blastulating from 12.7% to 5.8%, an action that offset the observed stimulatory effect of TGFα. PDGF reduces the affinity of EGF for its receptor (Bowen-Pope et al., 1983), and the same receptor modulates signal transduction by both TGFα and EGF (Derynck, 1986).

The main effect of TGFα in this study was to increase blastulation of 16-cell embryos from 8.6% to 40.6% (P < 0.025) while the apparent stimulatory effect of bFGF was not significant (P < 0.41). TGFα alone promoted blastulation in 23.8% of 16-cell embryos as compared with 47.9% when used in combination with bFGF. These data suggest synergism of the two growth factors even though statistical significance was not demonstrated. Both EGF and bFGF have been isolated in uterine secretions (Huet-Hudson et al., 1990; Brigstock et al., 1989).

Our results corroborate studies conducted on murine embryos, which also demonstrated that the first observable effect of EGF on mouse embryos occurs shortly after the time of compaction (Wood and Kaye, 1989). Receptors for EGF/TGFα have been identified on the surface of early murine morulae and blastocysts (Paria and Dey, 1990), and EGF stimulation of the early blastocysts induced expression of the cell cycle specific proto-oncoprotein fos (Adamson, 1990).

The present experiments have demonstrated that, in our defined in vitro system, PDGF was able to signal embryonically controlled development during the 8-cell stage. In this regard, PDGF mimicked the effect of the oviductal environment by stimulating continued development of the early bovine embryo. Alternatively, supplemental PDGF may have substituted for that which is normally synthesized by the embryo itself (Rappolee et al., 1988), and hence overcame a transcriptional block imposed by standard culture conditions. TGFα, perhaps aided by bFGF, signalled the continuation of embryonically controlled development and, in part, simulated the effect of the uterus on compaction and blastocyst formation.

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References


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