Insulin and insulin-like growth factor I (IGF I) in early mouse embryogenesis

RADAN SPAVENTI, MARIASTEFANIA ANTICA and KREŠIMIR PAVELIĆ
Ruder Bošković Institute, 41001 Zagreb, Bijenicka 54, P.O. Box 1016, Yugoslavia

Summary

Growth factors have an important role in the regulation of cell growth, division and differentiation. They are also involved in the regulation of embryonic growth and differentiation. Insulin and insulin-like growth factor I (IGF I) play an important part in these events in the later stages of embryogenesis, when organogenesis is completed. In this study, we are presenting evidence that insulin and IGF I are also secreted by embryonic tissues during the prepancreatic stage of mouse development. We found measurable amounts of insulin and IGF I in 8- to 12-day-old mouse embryos. We also showed that embryonic cells derived from 8-, 9- and 10-day-old mouse embryos secrete insulin, IGF I and/or related molecules. Furthermore, the same growth factors, when added to the culture of 9-day-old mouse embryonic cells, stimulate their proliferation. These results lead to the conclusion that insulin can stimulate the growth of embryonic cells during the period when pancreas is not yet formed, which is indirect evidence for a paracrine (or autocrine) type of action.

Key words: insulin, IGF I, mouse embryogenesis.

Introduction

Growth control can be mediated directly either by insulin, which can bind to the specific receptors and IGF receptors type I (Hill and Milner, 1985), or by the locally secreted IGF I (autocrine and paracrine growth control) (D’Ercole et al. 1980). Indirect regulation is mediated by insulin (Brinsmade and Liggins, 1979; Philips and Vassilopoulou-Sellin, 1980; Binoux et al. 1980, 1982) and placental lactogen (Hill et al. 1979) stimulation of IGF I secretion from the liver (and possibly kidney). Insulin and insulin-like growth factor I (IGF I) can also stimulate growth and differentiation of embryonic tissues grown in culture, depending upon cell types and developmental stage (Ridpath et al. 1984; Puro and Agardh, 1984; Ewton and Florini, 1981; Pratten et al. 1988; Cooke and Nicoll, 1984; DePablo et al. 1985). Travers et al. (1989) showed that explanted rat embryos require insulin for normal development between 9.5 and 11.5 days. Also, Sadler et al. (1986) presented data about somatomedin inhibitors from diabetic rat serum that alter growth and development of 9- to 10-day-old mouse embryos in culture. These in vitro studies suggest a possible role of insulin-like molecules as growth mediators during development. If this is so, the question is whether these peptides mediate fetal growth during the whole gestation period or just during the second half of it, when all prerequisites (growth hormone secretion, mature β cells, developed circulatory system, etc.) exist. Although there is some indirect evidence that insulin and IGF I could act at a time when the organogenesis is not completed, this still remains a matter of question.

The aim of this study was to investigate the role of insulin and IGF I at early stages of mouse embryogenesis and to find out whether these peptides are detectable in the 8- to 12-day-old embryos. We also tested the effects of insulin and IGF I on primary cultures of mouse embryonic cells, as well as the capability of these cells to secrete insulin and IGF I.

Materials and methods

Embryos

Inbred mice 4–6 weeks old of the CBA/H Zgr strain were obtained from pedigree lines maintained at the Ruder Bošković Institute (Zagreb, Yugoslavia) animal facility. Fetal mice were obtained from timed matings as previously described. The day of finding a vaginal plug was designated as 1st day of embryonic development (Sadler, 1979). 8- to 12-day-old mouse embryos were used. On the prescribed day, embryos were removed from the uterus and placed on a watch glass containing sterile cold PBS. Under a dissecting microscope, embryos were separated from extraembryonic tissues including the yolk sac.

Homogenates of embryonic tissue

Whole embryos (from at least 3 mice) were homogenized in an all-glass Potter-Elvehjem homogenizer. The homogenates were dissolved in 0.1 M-PBS and centrifuged at 70,000 g for 1 h at 4°C. The supernatants were used for insulin, C-peptide and IGF I determination. All experiments were done in triplicate. In some experiments, uterus homogenates of the same mice were used as controls.

Serum-free medium

This was prepared by supplementing pure Dulbecco-modified
Eagle's medium (DMEM) with EGF (50 ng ml⁻¹; Collaborative Research) and transferrin (25 µg ml⁻¹, Sigma).

**Primary cultures of embryonic cells**

These were prepared according to the method of Todaro and Green (1963). After fine mincing of the whole embryos, they were finally disaggregated with 0.25 % trypsin (Serva). After three subsequent washings in the cold with Hank's solution the cells were resuspended in DMEM containing 10 % FCS and incubated for 1 h at 37°C. The cells were counted, adjusted at a concentration of 10^5 cells per well and seeded in either 24-well plates at a final concentration of 10^5 cells per well or in 60 mm Petri dishes at 3x10^5 cells per dish.

After cultivating the cells in the 24-well plates for 3 days in DMEM with 10 % FCS, we replaced it with serum-free medium with the addition of insulin (10 µ.u. ml⁻¹; Novo Industrii) or IGF I (10 ng ml⁻¹; AmGen). In the control wells, only serum-free medium was added. Three days later the number and viability of cells was determined by trypan blue exclusion test. The results concern only the viable cells.

The concentrations of insulin and IGF I were assessed in the conditioned medium of the cells cultivated in Petri dishes. These cultures were first incubated in DMEM with 10 % FCS for 3 days. After washing the cultures three times with PBS, the serum-free medium was added and left for the next 3 days. The amount of growth factor in serum-free medium was measured by specific RIAs.

**Radioimmunochemical assays (RIAs)**

The concentrations of insulin, IGF I and C-peptide in tissue homogenates and culture supernatants were measured by means of specific RIAs. Insulin was measured according to Hunter and Greenwood (1982) with a commercial kit (Boris Kidrič Institute). IGF I was measured according to Daughaday et al. (1982) with a commercial kit (Amersham). C-peptide was measured according to the method of Helding (1975) using a commercially available kit from Byk Malinckrodt.

**Protein concentration**

This was measured in tissue homogenates by the method first described by Lowry et al. (1951).

**Statistical analysis**

This was performed by the Student's t-test.

**Results**

**Insulin and IGF I activity in embryonic tissue**

Fig. 1A shows the level of insulin in embryo homogenates (8- to 12-day-old mouse embryos). In all the tested samples, measurable amounts of this growth factor were found, although at this time the β cells, which are the only source of insulin in the adults, are not detectable. Already in the 8-day-old embryos there was a measurable insulin activity which reached its maximum level in the 9-day-old embryos (277 µ.u. mg⁻¹ proteins). On the 12th day of gestation, the insulin activity decreased to 50 µ.u. mg⁻¹ proteins. The present data are the results of three experiments.

In order to show that the measured activity was the result of insulin synthesis, we investigated the presence of C-peptide, the secondary product of this process. As shown in Fig. 1A (insert), the C-peptide was present in all the measured samples. The relation between the amount of this peptide and insulin was constant, giving indirect evidence for insulin-derived activity.

In the same samples (8- to 12-day-old mouse embryos), the concentration of IGF I was measured by specific RIA. As can be seen in Fig. 1B, the concentration of IGF I varies between different days of development in the range of 0.425–1.818 i.u. mg⁻¹ proteins. The highest values were detected on day 12 (1.818 i.u. mg⁻¹ proteins) and day 9 (1.583 i.u. mg⁻¹ proteins). The lowest amount of IGF I 0.425 i.u. mg⁻¹ proteins, was measured on day 10.

**Insulin and IGF I activity in primary cultures of embryonic cells**

Indirect evidence for the hypothesis that embryonic cells from the postimplantation stages of mouse development secrete insulin and IGF I (and/or related molecules) was provided by an in vitro experiment. Primary cultures of embryonic cells derived from 8-, 9- and 10-day-old mouse embryos were established as described in Materials and methods. After growing the cells for 3 days in serum-free conditions, their supernatants were collected and tested for the presence of the
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Days in culture

B

Days of gestation

Fig. 2. Insulin (A) and IGF I (B) concentrations in the supernatants of cells derived from 8-, 9- and 10-day-old mouse embryos, and normal mature mouse fibroblasts. In the supernatants of mouse fibroblasts there were no measurable insulin activity (A, control).

insulin and IGF I. We found that insulin and IGF I were present in all samples of conditioned medium.

Insulin

Insulin concentration in the supernatants of cells derived from 8- and 9-day-old mouse embryos was 1.31 μi.u. 10^{-5} cells and 0.8 μi.u. 10^{-5} cells, respectively. Even more insulin activity (5.45 μi.u. 10^{-3} cells) was measured in the culture supernatant of cells derived from 10-day-old embryos (Fig. 2A). As a control, the insulin concentration in the supernatant of normal fibroblasts, cultured under the same conditions was assessed. On the third day of culture, in the serum-free medium, there was no measurable insulin activity.

IGF I

IGF I concentration in the medium of mouse embryonic cells was elevated in comparison to cultures of normal mouse fibroblasts. The concentration of IGF I in the culture media of the cells derived from 9- and 10-day-old embryos was 0.109 i.u. per 5×10^{6} cells and 0.125 i.u. per 5×10^{6} cells, respectively. The culture supernatants of cells derived from 8-day-old mouse embryos contained 0.048 i.u. per 5×10^{6} cells IGF I, which is very close to the concentration in the control medium in serum-free fibroblasts cultures (0.05 i.u. per 5×10^{6} cells) (Fig. 2B).

The absence of insulin secretion in control fibroblasts (Fig. 2A) and the demonstration of IGF I production by these cells (Fig. 2B) gives additional proof for the specificity of the radioimmunoassay without any measurable cross-reactivity of insulin and IGF I.

Fig. 3. The number of cells in cultures derived from 9-day-old mouse embryos 3 days after the addition of either insulin (10 μi.u. ml^{-1}) (A) or IGF I (10 ng ml^{-1}) (B).

The influence of insulin and IGF I on primary cultures of embryonic cells

We further examined whether insulin and IGF I can stimulate the proliferation of mouse embryonic cells in vitro.

Since our previous results have shown that the tissue homogenates of 9-day-old embryos contain the highest concentration of IGF I and insulin, these embryos were selected for the further analysis. The cells derived from 9-day-old embryos were cultured in serum-free conditions. Either insulin or IGF I was added to these cultures at physiological concentrations, 10 i.u. ml^{-1} and 10 ng ml^{-1}, respectively. 3 days later the cells were counted. The number of cells was significantly higher in the cultures with either insulin or IGF I as compared to the control cultures where cells were grown without the investigated growth factors (Fig. 3). The number of cells in the group where insulin was added was 47,000 cells ml^{-1} (P<0.02) and in the one where IGF I was added 45,000 cells ml^{-1} (P<0.01). In the control group there were 22,000 cells ml^{-1}.

Discussion

It is still not clear whether embryonic cells in the early stages of development secrete insulin and if this peptide stimulates their proliferation. However, there is some evidence that insulin has a physiological role during embryogenesis. Namely, it has been shown that insulin stimulates growth of embryonic cells in vitro. Furthermore, De Pablo et al. (1982) demonstrated the presence of immunoreactive insulin in prepancreatic, 2-day-old chicken embryos which was required for their development (De Pablo et al. 1985). Miller (1988) has reported that stage IV oocytes from Xenopus laevis increase RNA, protein and glycogen synthesis in response to extracellular insulin. In addition, a similar role of insulin was also shown for some embryonal carcinoma (EC) cell lines, which, can, in spite of their tumorigenic nature, serve as an experimental model for the investigation of postimplantation stages of development (Martin, 1980). Physiological concentrations of insulin stimulates proliferation of embryonal carcinoma cell line F9 by binding to its own receptors (Nagarajan and Anderson, 1982). Undifferentiated PC13 EC cells do not express insulin receptors, but their differentiation in the presence of the retinoic acid results in the...
appearance of specific surface binding sites over which insulin increases DNA synthesis and proliferation (Heath et al. 1981). Another cell line, teratoma-derived line 1246-3A, produce a polypeptide growth factor tentatively called IRF (insulin-related factor), which is structurally and biochemically similar to pancreatic insulin but different from IGF I and IGF II (Yamada and Serrero, 1988). The same authors showed that IRF acts as an autocrine growth factor for these cells.

Since teratoma and teratocarcinoma cell lines produce several hormones and growth factors that have also been found in the embryo (D’Ercole et al. 1980; Adamson, 1982; Rizzino, 1985), it is reasonable to assume that insulin secreted by them may also be a part of the physiological growth regulatory system during embryonic development. One explanation of these findings is that in the pluripotent cells the insulin gene is active, while later, after differentiation and specialization, it remains active only in some differentiated tissues like the β cells of the pancreas, the yolk sac and possibly liver of the rat embryos (Muglia and Locker, 1984; Kimberley et al. 1989).

Recent results indicate that insulin is necessary for embryonic development from the early-somite stage (Travers et al. 1989). This hormone is also important for the normal growth of mouse conceptuses from the 2-cell embryo to the blastocyst (Rappolee et al. 1989; Harvey and Kaye, 1989). The discovery of active insulin receptors at these same stages of development support these results (Untermann et al. 1986). Since it is known that insulin does not cross the placenta (Adam et al. 1969; Kalhan and Adam, 1975) and that insulin secretion from the yolk sac starts about the same time as pancreas is developed (14th day in the rat) (Muglia and Locker, 1984; Kimberley et al. 1989), the question is what is the source of this peptide before that period? None of the results give a conclusive answer to this question.

In the present study, we show that insulin is secreted by non-extraembryonic mouse tissues. We found that there are measurable amounts of insulin in the embryonic tissues of 8- to 12-day-old mouse embryos. Since the β cells, which are the only source of insulin in the adult mouse, could be detected on the 11th day of gestation (Rall et al. 1973), the insulin detected before day 11 could be defined as extrapancreatic. The presence of this ‘extrapancreatic’ insulin was confirmed by measuring the levels of C-peptide, which is an indirect measure of normal insulin synthesis. Insulin detected on the 11th and 12th day could also be of pancreatic origin.

Further support for the hypothesis that embryonic cells secrete insulin at these early stages of embryogenesis was provided by in vitro experiments. The cells from primary cultures derived from 8-, 9- and 10-day-old mouse embryos secrete insulin and/or some related molecules. There is the possibility that some of these cells differentiate during the cultivation period and therefore secrete insulin. However, although it is known that whole embryos in culture could differentiate to a certain degree (New, 1978; Sadler, 1979), this is less likely for the cell suspensions because of the different milieu and growth conditions. This is supported by the cell morphology at the end of the cultivating period (data not shown).

Since the 9-day-old embryos contain the highest concentration of insulin, we tested the effect of exogenous insulin on the number of primary cultured embryonic cells. We show that physiological concentrations of insulin increase the number of cells significantly more than in the control cultures (embryo cells grown in un-supplemented serum-free medium).

These results lead us to the conclusion that insulin is secreted during the period of early embryogenesis when pancreas is not yet formed by the embryonic cells themselves, and that it might play an important role in their growth regulation.

IGF I is a polypeptide thought to be involved in the stimulation of somatic growth. Although its role in embryogenesis has not been elucidated, it has been shown that mouse fetal tissues at the stages of organogenesis (9–12 days of gestation) possess cell membrane receptors of relatively high affinity for IGF I (Smith et al. 1987). Also, the embryonal carcinoma cells (PC13) express IGF receptors and secrete binding protein into the medium (Heath and Shi, 1986; Smith et al. 1987). D’Ercole and co-workers (1980) investigated the production of IGF I in mouse embryo organ explants. They found that the intestine, heart, brain, kidney, liver and lung, but not the placenta, of 17-day-old mouse embryo secrete immunoreactive IGF I in vitro. In the same study they detected this growth factor in the media of fetal liver explants from day 11 embryos.

In this paper, we give evidence that IGF I is produced in the mouse embryo as early as the 8th day of gestation. The concentration of IGF I in media in which the embryonic cells were cultured was significantly higher than in the culture containing control, mature fibroblasts.

It seems that the synthesis and release of peptide growth factors, among them insulin and IGF I, might be the property of most, if not all, embryonic cells and that they act locally like autocrine and paracrine, but not as endocrine growth signals.

The authors would like to thank Drs Lidija Šuman, J. Pavelić, S. Marušič-Galešič and S. Levnan for critical evaluation of the manuscript.

This research has been undertaken with a financial contribution by the Commission of the European Communities Directorate-General for Science, Research and Development International Scientific Cooperation.

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(Received 10 November 1989)