Well-defined growth factors promote cardiac development in axolotl mesodermal explants

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Summary

The effect of growth factors on the formation of cardiac mesoderm in the urodele, Ambystoma mexicanum (axolotl), has been examined using an in vitro explant system. It has previously been shown that cardiac mesoderm is induced by pharyngeal endoderm during neurula stages in urodeles. In this study, explants of prospective cardiac mesoderm from early neurula stage embryos rarely formed beating cardiac tissue in culture. When transforming growth factor beta-1 (TGF-β1) or platelet-derived growth factor BB (PDGF) was added to such explants, the frequency of heart tissue formation increased markedly. The addition of other growth factors to these explants did not enhance cardiac mesoderm formation. The addition of basic fibroblast growth factor (bFGF) to prospective heart mesoderm derived from later stage embryos resulted in a decreased tendency to form cardiac tissue. These results suggest that growth factors analogous to TGF-β1, PDGF, and bFGF may regulate the initial stages of vertebrate cardiac development in vivo.

Key words: amphibian embryo, axolotl, cardiac induction, fibroblast growth factor, mesoderm, platelet-derived growth factor, transforming growth factor-beta.

Introduction

The vertebrate heart develops from embryonic mesoderm. The process of cardiac development has been studied most extensively in amphibian species. Fate map and embryonic explant studies have identified the location of cardiac precursor cells in several amphibian species. These studies have established that during early neurula stages amphibian cardiac primordia are paired dorsoanterior structures which migrate ventrally and ultimately fuse in the ventral midline (Chuang and Tseng, 1957; Jacobson, 1960; Jacobson, 1961; Jacobson and Duncan, 1968; reviewed by Jacobson and Sater, 1988). The results of explant experiments have also shown that amphibian cardiac development requires an inductive interaction. The timing of cardiac induction varies among amphibian species: it is completed by late gastrula stages in the anuran Xenopus laevis (Sater and Jacobson, 1989), yet in the urodele Taricha torosa, induction is not completed until late neurula stages (Jacobson and Duncan, 1968). The likely source of a cardiac inducing substance in urodeles, as determined by explant experiments, is the pharyngeal endoderm (Fullilove, 1970). This inducing substance has not yet been identified.

Cardiac differentiation is also regulated by the presence in vivo of inhibitory signals. A variety of explant experiments have suggested that certain tissues (especially neural cells) release an inhibitory signal which prevents cardiac differentiation (reviewed by Jacobson and Sater, 1988; Smith and Armstrong, 1990). This inhibitory signal has not been identified.

Polypeptide growth factors are likely candidates as initiators of inductive interactions in embryonic development. The presence of a variety of growth factors in vertebrate embryos have been demonstrated, including: platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor, nerve growth factor, and members of the transforming growth factor beta (TGF-β) family of molecules (Nexo et al. 1980; Adamson and Meek, 1984; Wilcox and Derynck, 1988; Miller et al. 1989; Lyons et al. 1989; Ruiz i Altava and Melton, 1989; Akhurst et al. 1990; Thomsen et al. 1990; reviewed by Whitman and Melton, 1989). A variety of experiments have suggested that FGF and a TGF-β-like molecule (possibly Activin B) are the natural substances that induce the formation of mesoderm (Slack and Forman, 1980; Slack et al. 1987; Kimelman et al. 1988; Kimelman and Kirschner, 1987; Rosa et al. 1988; Green et al. 1990; Thomsen et al. 1990; reviewed by Smith, 1989). Whether these factors or others regulate cardiac development is not known. The finding that mRNA for TGF-β1 is expressed within or adjacent to the cardiac
plate of murine embryos has suggested that this factor plays a role in cardiac development (Akhurst et al. 1990).

In this study, embryos from the species *Ambystoma mexicanum* (axolotl) were used to examine the potential role of growth factors in cardiac induction. Axolotl embryos are advantageous compared to other amphibian embryos because of their large size, easy availability, and slow pace of development (Armstrong and Malacinski, 1989). Early neurula stage axolotl pre-cardiac mesoderm rarely formed beating heart tissue in culture. When certain growth factors were added to such mesodermal explants, beating heart tissue formation was dramatically enhanced.

**Materials and methods**

**Embryonic explant preparation**

Axolotl embryos were obtained from the Indiana University Axolotl Colony. Embryos were stored at 18°C in 20% modified Steinberg’s Solution (MSS), pH 7.4 (Armstrong and Malacinski, 1989), and were staged according to the system described by Schreckenberg and Jacobson (1975). Embryos were de-jellied manually. Watchmaker’s forceps were used to remove the vitelline membrane from embryos. Pre-cardiac mesoderm with underlying ectoderm was isolated from neurula stage embryos using glass needles, eyebrow hair knives, and hair loops. All microsurgery was performed in agar-lined plastic culture dishes in 100% MSS with added penicillin G 100 units ml⁻¹, streptomycin sulfate 100 μg ml⁻¹, and fungizone 1.25 μg ml⁻¹. Embryonic explants were incubated for thirty minutes in 100% MSS with added antibiotics in culture dishes at 18°C. (Sater and Jacobson, 1989). During this incubation period, the underlying ectoderm formed an epithelial vesicle surrounding the explanted mesoderm. Epithelial vesicles were found to promote the survival of enclosed embryonic tissues. In some cases, growth factors were added to incubation solutions. Explants were then transferred in incubation solutions to cover slips to make hanging-drop cultures. Explants were maintained at 18°C and were observed daily for 30 days using a compound microscope.

**Growth factors**

Growth factors were added to incubation solutions that bathed explants of mesoderm. Human recombinant TGF-β1 (Genentech, Inc.), human recombinant PDGF BB (Chiron Corp.), human recombinant basic FGF (Chiron Corp.), human recombinant EGF (Genzyme), all-trans retinoic acid (Sigma), and porcine insulin (Collaborative Research) were added at the concentration indicated in the text and figures. Bovine serum albumin (final concentration 0.1 mg ml⁻¹) was added to the incubation solutions containing TGF-β1, bFGF, PDGF, and EGF. Each explant that was exposed to growth factor was matched with a control explant obtained from pre-cardiac mesoderm derived from the contralateral side of the embryo. Control explants were incubated in 100% MSS with added antibiotics and the appropriate diluents.

**Histology**

Explants for histological analysis were fixed in 4% paraformaldehyde for 1–2 h, washed in 50% ethanol, dehydrated to 100% ethanol, placed in xylenes, then impregnated with paraplast. Eight micron sections were cut and dried onto gelatin-subbed glass slides. Sections were stained with toluidine blue.

**Statistical Analysis**

As noted above, mesodermal explants were observed for 30 days for the presence of beating cardiac tissue. Explants which exhibited cell autolysis within the 30 day observational period were excluded from statistical analysis. Paired experimental and control explants, obtained from contralateral pre-cardiac mesoderm, were compared using Chi-square analysis or Fisher’s Exact Test (when Chi-square analysis was inappropriate).

**Results**

**Timing of cardiac induction in axolotls**

The timing of cardiac induction in axolotls was determined by examining anterolateral mesodermal explants for the formation of beating heart tissue. Mesodermal explants were isolated from embryos at various developmental stages within vesicles of ectodermal epithelium and were maintained in hanging-drop cultures (Fig. 1). Explants were initially darkly pig-

![Fig. 1. Schematic representation of the experimental method. Anterolateral mesoderm and overlying ectoderm was isolated from early neurula stage axolotl embryos and exposed to various reagents in agar-lined culture dishes. After an incubation period explants spontaneously formed vesicles and were then placed in hanging drop cultures (see text for details).](image-url)
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merited but became translucent after 5 to 6 days in culture. Beating cardiac tissue was easily recognized as a small clump of cells that contracted synchronously at a rate of 20 to 30 beats per minute (Fig. 2). This rate of contraction is consistent with amphibian myocardium (Jones, 1967; Jones and Shelton, 1963). Early neurula (stage 14) mesodermal explants rarely formed beating cardiac tissue, while late neurula (stage 18) explants often formed such beating tissue (Fig. 3).

When pharyngeal endoderm was included in mesodermal explants from early neurula-stage embryos, heart tissue formed frequently (Fig. 3). In paired explant experiments, stage 14 mesodermal explants with added endoderm formed beating heart tissue in 63% of cases (12/18), compared with 0% (0/19) of control explants without added endoderm. The effect of added endoderm on the rate of heart tissue formation was significant by Chi-square analysis (Chi-square with continuity correction 14.7, P=0.0001). The mean time before beating was observed in endoderm-containing explants was 9.1±1.1 (s.e.) days. In addition, explants at all neurula stages developed larger and more structured hearts when endoderm was included.

Addition of growth factors to early neurula-stage mesodermal explants

The effect of growth factors on cardiac induction was examined using pre-cardiac mesodermal explants from early neurula stage axolotl embryos. Stage 14 mesodermal explants, which rarely formed beating heart tissue in culture, were used to assay the cardiac inducing potentials of several growth factors. Explants were bathed in solutions containing growth factors and were observed daily for the formation of heart tissue. The concentrations of growth factors used were based in part on experiments in which blastula stage animal cap explants were used as a model of mesodermal induction in Xenopus (Ruiz i Altaba and Melton, 1989; Green et al. 1990). The growth factors used in this study have all been localized in the embryonic tissues of vertebrates. In general, the amino acid sequences of the growth factors tested here are highly conserved among vertebrate species, allowing for the use of mammalian factors with amphibian tissues.

When stage 14 mesodermal explants were bathed in incubation solution containing 30 ng ml⁻¹ of TGF-β₁, 59% (20/34) formed beating heart tissue compared with 0% (0/34) of paired controls (Figs 2 and 4). This result was highly significant by Chi-square analysis (Chi-square with continuity correction 25.6, P=0.0001). The mean time before beating was observed in mesodermal explants exposed to TGF-β₁ was 11.3±1.2 (s.e.) days. When 50 ng ml⁻¹ PDGF BB was included in the incubation solution, 41% (12/29) of explants formed beating tissue compared with 0% (0/29) of paired controls (Chi-square with continuity correction 12.7, P=0.0004). The mean time before beating was observed in explants exposed to PDGF BB was 12.8±2.1 days. Incubation solutions containing 50 ng ml⁻¹ of EGF increased heart formation to 22% (7/32) compared with 6% (2/32) of paired controls.

Fig. 2. Early neurula stage axolotl mesodermal explants in epithelial vesicles after 2 weeks in culture. A, Control explant without beating cardiac tissue formation. B, Explant exposed to TGF-β₁ (30 ng ml⁻¹). Beating cardiac tissue formation was observed at the site indicated by the arrow. C, Explant containing pharyngeal endoderm. The beating cardiac tissue in the endoderm-containing explant (arrow, panel C) was larger and more developed morphologically than the comparable beating tissue in the explant exposed to growth factor (arrow, panel B). Scale bar, 250 μm.
The effect of EGF on beating tissue formation in mesodermal explants was not significant by statistical analysis (Fisher's Exact Test, 2-tailed, P>0.05). The mean time to beating for EGF-exposed explants was 13.0±3.5 days.

Stage 14 mesodermal explants incubated in salt solution containing 50 ng ml\(^{-1}\) of basic FGF did not form beating heart tissue in any of 23 explants (0/23) (Fig. 4). Insulin, at a concentration of 600 ng ml\(^{-1}\) had no effect on heart formation in this assay (0/25). Retinoic acid was also ineffective at promoting heart development at a concentration of 1 \(\mu\)M (3%, 1/29).

Fate map and embryonic explant studies have demonstrated that posterior mesoderm from neurula stage urodele embryos does not usually form cardiac tissue (reviewed by Jacobson and Sater, 1988). To assess whether TGF-\(\beta\) could respecify posterior mesoderm to form heart, Stage 14 posterior mesodermal explants were examined for their ability to form beating heart tissue in hanging-drop culture. In no cases did posterior mesodermal explants cultured in salt solution (0/23) or in TGF-\(\beta_1\) 30 ng ml\(^{-1}\) (0/23) form beating heart tissue in culture.

**Histologic appearance of explants**

Early neurula mesodermal explants were maintained in culture for 2 weeks then were fixed and stained as noted above. Mesodermal explants incubated in salt solution were composed largely of thin cells immediately within the epithelial vesicle, and loose collections of fibroblast-like cells (Fig. 5). These cell types have been referred to as mesothelium and mesenchyme, respectively, by Green et al. (1990). Mesodermal explants exposed to TGF-\(\beta_1\) sometimes exhibited tightly-packed spherical collections of cardiomyocytes contained within a thin
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Fig. 5. The histologic appearance of early neurula stage mesodermal explants fixed after 2 weeks in culture. A, control mesodermal explant. Note the thin sheet of mesothelium-like cells (me) adjacent to the epithelial vesicle. B, Mesodermal explant exposed to TGF-β. Note the circular collection of tightly packed cardiomyocytes (cm), contained within a thin-walled sac. Scale bar, 180 μm.

sac which, in turn, was contained within the thick-walled epithelial vesicle. Other mesodermal explants exposed to TGF-β were composed of small collections of cardiomyocytes within the epithelial vesicle.

Discussion

We have examined the effect of growth factors on axolotl cardiac induction using a mesodermal explant system. Previous studies have suggested that cardiac induction in amphibians results from the release of a signal from endoderm which influences mesoderm to differentiate into myocardium (reviewed by Jacobson and Sater, 1988). The timing of this inductive interaction appears to vary between amphibian species, occurring earlier in anurans than in urodèles (Sater and Jacobson, 1989). Recently, Smith and Armstrong (1990) reported that cardiac specification in axolotls occurs during early neurula stages, using isolated mesodermal explants without the addition of inducing substances. In their study, more stage 14 mesodermal explants formed beating heart tissue (20%) than in our work, however, this may be explained by their use of a different staging system in which stage 14 is defined by slightly more mature embryos.

Taking advantage of the fact that cardiac induction is not completed in early neurula stage axolotl embryos, we tested the ability of various growth factors to enhance heart formation in mesodermal explants. Two well defined growth factors enhanced heart formation in this in vitro system. Undifferentiated anterior mesoderm, when exposed to TGF-β or PDGF BB, formed beating heart tissue. Although our results do not prove that these factors induce cardiac tissue in vivo, both TGF-β and PDGF have been found in neurulating vertebrate embryos. One interpretation of our results is that TGF-β and PDGF replicate the effects of a natural inducer molecule, stimulating a pattern of gene expression in undifferentiated mesoderm that culminates in the formation of heart. However, addition of TGF-β or PDGF to mesodermal explants did not completely compensate for the removal of pharyngeal endoderm: cardiac tissue that developed in growth factor-exposed mesodermal explants was smaller, simpler in structure, and had delayed onset of beating compared with endoderm-containing explants.

The assay system used in this study, the observation of rhythmically beating heart cells, examines a late stage in cardiac development. A large variety of structural changes are required to transform an undifferentiated mesodermal cell into a beating heart cell. In our observation system, 10 to 14 days were required to observe the effects of growth factors. The prolonged nature of this system affects the interpretation of the data presented in several ways. First, it is possible that some of the ineffective growth factors tested may stimulate the synthesis of some but not all of the proteins required for spontaneous beating. Second, it is not clear that TGF-β and PDGF stimulation of mesoderm establishes a pattern of gene expression which culminates in heart formation. These factors might simply enhance a pre-established pattern of cardiac gene expression which results in sufficient protein synthesis for spontaneous beating.

The fact that more than one growth factor was effective in promoting beating heart formation raises further questions about cardiac induction in amphibians. Each growth factor may cause intracellular metabolic alterations that reproduce the effects of the natural inducer, such as activation of an intracellular kinase. Alternatively, one of the growth factors tested may be highly homologous to the native inducer and the other effective factor may simply increase production of the in vivo inducer. For example, PDGF may stimulate production in mesoderm of TGF-β which, in turn, is the natural inducing substance.

In this study, only anterolateral mesoderm from early neurula stage embryos was observed to form beating heart tissue in culture. When posterior mesoderm was
cultured in the presence of TGF-β1, beating tissue formation did not occur. This suggests that TGF-β1, at the concentration tested, was unable to respecify posterior mesodermal cell fate.

We found that bFGF inhibited myocardial development in axolotl mesodermal explants. During normal embryogenesis, pre-cardiac mesoderm is located adjacent to the neural plate during early neurula stages. As neurulation proceeds, pre-cardiac mesoderm migrates ventrally, away from the forming neural tube. Jacobson and Duncan (1968) noted that amphibian neural tissue released some inhibitory substance which inhibits beating heart tissue formation in mesodermal explants. Several studies have demonstrated the presence of FGF in developing vertebrate neural tissue (Gonzalez et al. 1990; Masearelli et al. 1987; Risau, 1986). It is therefore possible that FGF may be a natural inhibitor of cardiac induction.

The ability of bFGF to inhibit cardiac differentiation suggests that although cardiac specification is completed in many embryos by mid-neurulation, cardiac determination has not yet occurred. In fact, Smith and Armstrong (1990) concluded that axolotl cardiac induction required 2 signalling interactions: an initial signal that causes cells to begin differentiating into myocardium, and a second signal that leads to the organization of functional sarcomeric myofibrils. Development of organized myofibrils, which is necessary for the onset of rhythmic beating, may occur spontaneously after the initial inductive interaction if inhibitory signals are absent (such as in mesodermal explants). Given this two-step model of cardiac induction, our findings suggest that: (1) TGF-β1 or PDGF can replicate the effect of the natural first-step inducer and stimulate the development of functional myocardium in the absence of inhibitors (in explants), and (2) bFGF simulates the effect of the in vivo inhibitor and prevents the completion of myocardial cell differentiation in tissue that has already received the initial inductive signal. Exposure of stage 14 mesodermal explants to both TGF-β1 and bFGF did not result in an increased frequency of beating heart formation suggesting that TGF-β1 was unable to compensate for bFGF inhibition of cardiac differentiation.

The identification of highly specific early marker gene products of cardiac induction will be helpful to further examine the interactions that lead to heart formation. Unfortunately, many cardiac sarcomeric protein gene products are not uniquely expressed in embryonic cardiac tissue. No regulatory genes of cardiac development have been identified at this time. This axolotl mesodermal explant system, however, offers an approach to identifying early marker and regulatory gene products of cardiac induction.

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References


between peptide growth factors and homeobox genes in the establishment of antero-posterior polarity in frog embryos.


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