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POLYLYSINE STIMULATION OF ECTOPIC CARTILAGE FORMATION

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Abstract

Cartilage development, or chondrogenesis, is a process which involves the condensation of prechondrogenic mesenchymal cells, followed by the expression of the cartilage phenotype characterized by the biosynthesis of cartilage-specific, extracellular matrix components. We have previously shown that the cationic biopolymer, polylysine (PL), is able to stimulate chondrogenesis by embryonic limb bud mesenchymal cells in vivo. In this study, we have evaluated the chondrogenesis-stimulating activity of PL in vitro using an experimental system consisting of chick embryonic muscle explants, which were grafted onto the chorioallantoic membrane of chick embryos maintained in long-term shell-less culture. The muscle grafts were treated with PL by multiple direct injections of PL of 398 KD M_r. The phenotypic changes in the muscle grafts were assessed by histology, scanning electron microscopy, and analysis of the biosynthesis of extracellular matrix components. The microscopic observations revealed that a cartilage-like matrix was elaborated in the PL-treated grafts, as indicated by positive alcian blue staining and the presence of abundant matrix material morphologically similar to that in a typical cartilage. Cartilage matrix biosynthesis in the PL-treated grafts was further indicated by their increased cartilage-specific, extracellular matrix components.

Key words: polycation, biopolymer, chondrogenesis, tissue graft, chick embryo, histology, scanning electron microscopy, extracellular matrix.

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Introduction

The surface of eukaryotic cells generally carries a net negative charge, a result of the biochemical characteristics of the consortium of membrane proteins and carbohydrate moieties (Alberts, et al., 1989). These membrane constituents confer upon the cell its ability to interact specifically with other cells as well as with components of the extracellular matrix (Höök, et al., 1984; Hynes, 1987; Ruoslahti, 1988). Experimentally, this surface negative charge also permits the attachment of cells to positively charged surfaces in vitro, forming the basis for tissue and cell culture on modified polystyrene plastic (Jakoby and Pastan, 1979). Among the agents which have been applied to enhance this interaction to expedite the attachment of cells in vitro is the cationic polypeptide, poly-L-lysine (PL) (McKeehan and Ham, 1976). Thus, cells seeded onto surfaces which have been coated with PL generally show better attachment and subsequent growth. On the other hand, PL has also been used as a non-specific agglutinin for blood cells (Coakley, et al., 1985; Danon, 1963; Nevo, et al., 1955).

Compaction or condensation of a group of cells is a fundamental cellular activity underlying a large number of phenomena during development and morphogenesis, for example the compaction of the blastomeres of the early 8-cell developing embryo (Gilbert, 1988), the condensation of the prechondrogenic core of the developing limb (Ede, 1983; Amprino, 1984; Oster, et al., 1985; Thorogood and Hinchliffe, 1975), the cellular aggregation during formation of the otic capsule (McPhee, et al., 1987), and the dermal condensations involved in feather germ development (Stuart, et al., 1972), etc. These events, although not necessarily equivalent, are most likely mediated by the molecular interactions between specific cell surface components, a process which is ultimately important for the subsequent phenotypic or morphogenetic events (Edelman, 1986; Ekblom, et al., 1986).

Work in this laboratory for the last several years has addressed the involvement of cellular condensation in chondrogenesis, in particular whether artificial perturbation of this process may lead to altered chondrogen-
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sis. Using isolated chick embryonic limb mesenchymal cells cultured in vitro as an experimental system, we have studied the effect of PL on chondrogenesis (San Antonio and Tuan, 1986; 1987). In these experiments, PL was added to the culture medium bathing the cells instead of being used to coat the surface of the culture vessel; the rationale being that such an application would serve to enhance cell-cell interaction through general PL binding and bridging of the cells. Results from these studies indicate that PL dramatically enhanced chondrogenesis in vitro, in a dose- and time-dependent manner. Biosynthesis of sulfated proteoglycans and type II collagen, hallmarks of cartilage, is specifically stimulated. Furthermore, the effect of PL is strictly size regulated, i.e., only PL preparations of \( \geq 8 \) KD are effective, strongly suggesting that spatial cross-linking of cells by PL, which would indeed have a minimal molecular size requirement, is likely to be functionally involved in the stimulation of chondrogenesis by PL. Additional studies further suggest that cell surface heparan sulfate glycosaminoglycans may be the cellular counterpart to interact with the exogenous PL (San Antonio, et al., 1987). A recent report also presents data indicating that PL also acts on chondrocytes to enhance their matrix biosynthetic activities (Takeda, et al., 1989).

The present study has been undertaken to evaluate further the apparent chondrogenesis stimulating activity of PL in vivo. The goal is to introduce PL into a living tissue, and to observe the subsequent effect on the tissue phenotype, particularly with regard to cartilage formation. The system chosen for this study is embryonic muscle, which is excised and maintained as a viable tissue graft in vivo. This system is chosen for the following reasons: 1) intramuscular sites are known to permit ectopic chondrogenesis, for example by the implantation of demineralized bone matrix (Reddi and Huggins, 1972; Urist, 1965) and various factors or substances derived from the matrix (Bentz, et al., 1989; Wozney, et al., 1988); 2) this biological response strongly implies that muscle probably contains presumptive "chondroprogenitor cells", which may be induced by appropriate stimuli to undergo chondrogenesis; and 3) these cells should thus serve appropriately as candidate stem cells to test for the action of potential chondrogenesis inducing agents, such as PL. Finally, embryonic tissues were chosen since they should possess high morphogenetic potential, such that any phenotypic changes as a result of exogenous stimulation or induction would be easily detectable. In the present study, the effect of PL treatment has been studied in terms of histology and ultrastructure, and biochemically based on biosynthesis of extracellular matrix components.

Materials and Methods

Chick Embryos

Fertilized white Leghorn chicken eggs were obtained from Truslow Farms (Chestertown, MD), and incubated for the required periods of time in a forced-air, humidified commercial egg incubator at 37.5°C.

Preparation of Muscle Grafts

Chick embryos incubated for 12 or 15 days were used as muscle graft donors. The muscle explants were obtained by aseptically dissecting the pectoral or thigh muscles, which were cut into 2-5 mm³ pieces, and rinsed in physiological saline to remove all adherent skin and feather. For each donor embryo, muscle explants were routinely prepared from comparable contralateral regions for direct comparison (see below).

Embryo Hosts for Muscle Grafts

The muscle explants were grafted onto the chorioallantoic membrane (CAM) of chick embryos as described below. Chick embryos were maintained as shellless (SL) cultures as described previously (Jacenko and Tuan, 1986a, b; Ono and Tuan, 1986; Tuan, 1980; Tuan and Lynch, 1983). Briefly, the SL chick embryo cultures were prepared by aseptically placing the entire content of a fertilized egg minus the eggshell, after having been incubated for three days in ovo, into a sterile plastic sac suspended within a ring-stand. The culture was then covered with a Petri dish, and then incubated at 37.5°C in a humidified tissue culture incubator with...
constant air flow. These SL embryos developed in a manner similar to those incubated in ovo, with the exception that they became highly calcium deficient during late stages of development, owing to the absence of the eggshell, its primary source of calcium (Tuan, 1987). In these cultures, the CAM, a highly vascularized extraembryonic membrane used by many investigators for tissue grafting studies (McLachlan, 1981), developed as an extensive membrane sac covering the whole embryo, and was thus rendered highly accessible to tissue grafting and experimental manipulations (Figure 1). The muscle explants obtained as described above were rinsed with physiological saline, blotted gently to remove excess fluid, and placed onto the CAM of day-8 (total incubation period) SL embryos. In general, three to four grafts were placed onto the CAM of an SL embryo. The culture was then returned to the incubator for further development of the embryo. In some instances, the tissue grafts were removed with the underlying CAM after 5 days, and then re-grafted onto the CAM of another day-8 SL embryo and kept for an additional week.

**PL Treatment of Muscle Grafts**

The PL used for this study was a 398 KD preparation (Sigma Chemicals) and was prepared in physiological saline at a concentration of 12.5 µg/ml. PL was injected directly (50 µl volume, dose of 0.625 µg/injection) into the center of the muscle grafts using a 26G syringe needle as follows: 1) immediately after excision from the donor embryo and after rinsing in saline, the muscle explants were placed in Petri dish and injected; 2) after the explants had been placed on the CAM of the host embryo, PL was injected directly into the grafts every two days in a similar manner (total of three injections for a 5-day grafting on a single embryo host, and six injections for the 12-day grafting using two host embryos). Routinely, grafts placed on the same embryo were divided into experimental (injected) and control (uninjected or injected with saline alone) groups to observe directly the effect of the PL treatment. In some cases, controls consisted of grafts, which were all uninjected, placed on the same embryo host. The dose of PL chosen was calculated based on tissue size and injection volume to approximate the optimal range of PL concentrations shown to be active in vitro (San Antonio and Tuan, 1986).

**Histology**

Specimens were fixed in Hollande-Bouin's Fixative for one to two days, rinsed in water, dehydrated, embedded in Paraplast, and sectioned at 8 µm thickness. Histological staining was carried out as described in Humason (1967) and included: 1) hematoxylin-eosin (H-E) for general histology, 2) alcian blue at pH 1.0 for the detection of sulfated cartilage matrix, and 3) alizarin red for mineralization. The stained sections were examined using an Olympus BH2 microscope and photographed with Kodak Panatomic-X film.

**Scanning Electron Microscopy**

The tissue specimens were fixed for 2 h at room temperature in 2.5% glutaraldehyde in 0.13 M cacodylate buffer, pH 7.4, rinsed twice in the same buffer, post-fixed in 1% OsO4/cacodylate buffer for 1.5 h, rinsed three times in buffer, and then dehydrated through a graded series of ethanol. The specimens were critical point dried. Specimens were mounted with adhesive onto aluminum mounts, progressively fractured with a razor blade, and coated with gold in a Polaron sputter coater. Examination of the specimens was done using a JEOL 35C scanning electron microscope at an accelerating voltage of 25 kV.

**Biosynthesis of Cartilage Matrix**

Muscle grafts were removed after 5 or 12 days of maintenance on top of the CAM, rinsed in physiological saline, and placed into organ culture in Dulbecco's Modified Eagle's Medium, containing fetal bovine serum (10%) and antibiotics (penicillin/streptomycin). The organ cultures were kept in a tissue culture incubator at 37°C in a humidified 5% CO2 atmosphere for 24 h. To estimate the biosynthesis of sulfated glycosaminoglycans, [35S] sulfate (ICN Biochemicals; 5 µCi/ml of medium) was added to some of the cultures (San Antonio and Tuan, 1986). In other cultures, collagen was metabolically labelled by the addition of [3H]proline (ICN; 25 µCi/ml of medium); these cultures were also supplemented with β-amino proprionitrile (25 µg/ml) and ascorbate (50 µg/ml) to optimize collagen extractability and biosynthesis, respectively (Tuan and Lynch, 1983). At the end of organ culture, the grafts were rinsed for several times in physiological buffer, fixed in 10% trichloroacetic acid (TCA) at 4°C for 30 min, washed extensively in 10% TCA followed by water until all unincorporated radioactivity was removed from the tissue, and then lyophilized to dryness. The [35S] sulfate-labelled specimens were rehydrated and solubilized with Tissue Solubilizer (Fisher Chemicals), and incorporated radioactivity was determined by liquid scintillation counting and expressed per dry weight of the specimens. The [3H]proline-labelled samples were rehydrated and solubilized in sodium dodecyl sulfate (SDS) buffer, and analyzed by SDS polyacrylamide gel (6%) electrophoresis and fluorography for the analysis of collagen type synthesis as described previously (Tuan and Lynch, 1983). The relative abundance of collagen α1 and α2 chains was estimated by densitometric scanning of the fluorograph.

**Results**

The objective of the present study was to examine whether administration of PL would promote or induce cartilage formation in ectopic tissue sites, specifically chick embryonic muscle explants maintained as grafts on the CAM of a developing chick embryo host. The results presented here, based on both morphological and biochemical characterizations, strongly suggested that PL acted in vivo to elicit chondrogenesis in these crafted muscle explants.

**Muscle Grafts**

The set-up of the SL embryo for tissue grafting is
shown in Figure 1. In this set-up, the chick embryo developed within the plastic sac and the CAM extended to cover the entire surface of the embryo culture, thus permitting the placement of multiple tissue explants onto each embryo and thereby "normalizing" the external influences on these explants. Furthermore, this system also permitted the transferring of a tissue explant from one embryo to another, so that it was possible to extend the grafting period from 5 days to almost 2 weeks in some cases. Finally, multiple injection of the tissue graft was rendered easy by the direct accessibility of the CAM of the SL embryo.

In the first set of four experiments, a total of 72 muscle explants (including experimental and control) were grafted onto the CAM of SL chick embryo hosts. In experiment one, day-12 embryos were used as the
Figure 2. Histology of muscle grafts. A, B. General histology of the muscle graft explanted for 5 days on the CAM. The cross-sectional views revealed the maintenance of the fiber bundles (F) and architecture of the striated muscle. C-F. Areas of alcian blue positive staining (arrows) in various grafts (C, E - H-E staining to show histology; D, F - alcian blue staining). Note that alcian blue staining (D, F) was localized to the extracellular matrix surrounding large, round cells (E). G, H. Histology of muscle graft explanted for 12 days on the CAM (G - H-E staining; H, alcian blue staining), showing a prominent cartilage-like, alcian blue positive zone (arrows) which contain large round cells. I, J. Area in 5-day grafts negative for alcian blue (I - H-E staining; J - alcian blue staining). K, L. Endoderm of CAM showing highly localized alcian blue staining (arrows). Bar denotes 50 µm. Magnification identical for B-D, F-L.
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Table 1 External Morphology and Histology of Embryonic Muscle Grafts

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* The muscle grafts were examined in situ for external appearance (color, and extent of vascularization and incorporation into the CAM).

** Experiment number refers to separate experiments using identical protocol. Specimens were chosen randomly from these experiments.

† These grafts were not treated with PL and were placed as a group without adjacent PL-treated grafts on the CAM.

source of the muscle explants, and day-15 embryos for the other experiments. Thirty-three of these grafts remained at the end of the experimental period (5 days), i.e., on incubation day-13 of the host embryo, with injections of PL given at the beginning prior to grafting, and on the third and fifth days post-grafting. In general, there was no apparent difference between the muscle grafts derived from day-12 and day-15 embryos. The muscles of day-15 embryos were more easily dissected and were consequently used for the later experiments. Twenty-four randomly selected grafts were processed for histology, sectioned in toto, and the sections were stained with H-E, alcian blue and alizarin red per 300-400 µm of the tissue. Eight of the 24 specimens showed cellular necrosis and clearly failed to survive the 5-day grafting period. The 16 viable muscle grafts included 10 that were injected with PL and 6 that were uninjected. The external appearance of these grafts on the CAM is summarized in Table 1. In general, the viable grafts were usually characterized by a tan-to-beige coloration, with varying extent of vascularization and incorporation into the CAM, whereas necrotic tissues were invariably chalkish white, and appeared contracted with no incorporation into the underlying CAM.

**Histology**

Positive alcian blue staining was used as a criterion to assess the presence or the production of a sulfated extracellular matrix, a hallmark of the cartilage phenotype. The results presented in Table 1 showed that the PL-injected grafts consistently showed a significantly higher degree of alcian blue staining, whereas staining of the uninjected explants was considerably less. In addition, 90% of PL-treated explants were alcian blue positive compared to 50% of the uninjected controls. Interestingly, all uninjected grafts that were placed by themselves (i.e., without PL injection into neighboring or adjacent explants placed on the same CAM) were consistently alcian blue negative, suggesting some seepage of
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Figure 3. Morphology of embryonic muscle and cartilage as revealed by scanning electron microscopy. A. Pectoral muscle; B. thigh muscle; C. sternal cartilage; and D. epiphyseal growth plate cartilage. Abundant striated fibers (arrows) are seen in the muscle specimens, whereas cartilage is characterized by round cells (solid arrows) embedded in a substantial extracellular matrix (open arrows). Bars denote 10 µm.

PL might have occurred from the injected grafts to neighboring uninjected grafts when both grafts were placed on the same CAM. All specimens were negative for alizarin red, indicating that no mineralization occurred within these grafts.

The histology of the muscle grafts is illustrated in Figures 2A-L. Several salient features of the histology are: 1) the striated architecture of the skeletal muscle was largely maintained in the muscle grafts on the CAM (Figs. 2A, B), although not within the entire explant; 2) areas of alcian blue positive staining were found within various interior regions of the grafts (Fig. 2C, E - H-E staining; Fig. 2D, F - alcian blue staining); and 3) other areas that did not stain with alcian blue (Fig. 3I, J) appeared to retain more of the striated muscle architecture. Overall, the alcian blue stained regions were detected within the core and constituted only a small portion of the graft. Interestingly, the endoderm of the CAM that served as the graft site consistently stained with alcian blue (Fig. 3K, L), probably due to the presence of sulfated glycosaminoglycans within the basal lamina of the endoderm.

Upon visual inspection, muscle grafts maintained for 12 days on the CAM appeared to be much more condensed than the 5-day grafts, i.e., the tissue mass contracted and usually became darker, perhaps as a result of the continued vascularization and incorporation into the underlying CAM, and occasionally a hard, central core of solid mass was present. Histologically, alcian blue positive staining was again evident (12 specimens examined), with the most intense staining generally being associated with the central core of the graft (not shown). In rare instances, as shown in Fig. 3G, H, significant alcian blue staining was seen in the central core with almost typical cartilage histology, i.e., round cells surrounded by an extensive dense blue matrix (also see below for analysis by scanning electron microscopy).

Taken together, these observations indicated that morphological, and perhaps phenotypic changes might have taken place within the muscle grafts as a result of...
Figure 4. Morphology of muscle grafts maintained for 5 days on CAM as revealed by scanning electron microscopy. A, B, D: PL-treated graft; C: Control graft. A. Low magnification view of the entire muscle graft showing fracturing of the specimen (arrows) to expose the internal morphology. B, C. Disorganized fibers (arrows) seen in the muscle grafts with (B) or without (C) PL-treatment. D. Occasional presence of cells (arrows) with round morphology in the core of a PL-treated graft. These cells were seen more frequently in the PL-treated grafts than in control grafts. Bar denotes 100 µm in A and 10 µm in B, C, and D.

PL treatment. The alcian blue positive staining strongly suggested that a cartilage phenotype was present in the PL-treated grafts.

Scanning Electron Microscopy

In a second set of experiments, muscle explants from day-15 embryos were harvested after 5 and 12 days of maintenance as grafts on the CAM of SL embryos (see Materials and Methods). Grafts which were viable based on visual inspection (see above) were processed for scanning electron microscopy as described in Materials and Methods. For the purpose of comparison, samples of two tissue types, i.e., cartilage (sternum and tibial epiphyseal growth plate) and muscle (pectoral and thigh) were also dissected from a day-15 embryo and similarly processed. The general morphology of embryonic cartilage and muscle is shown in Figure 3. The presence of abundant striated fibers were evident in the muscle (Fig. 3A, B), which was used to prepare the grafts. On the other hand, both epiphyseal and sternum cartilage specimens were characterized by the substantial, prodigious extracellular matrix within which round chondrocytes were embedded (Fig. 3C, D). These distinct morphological differences between muscle and cartilage served as reference to evaluate the putative presence of cartilage phenotype in the PL-treated grafts as suggested by the histological features described above (see Fig. 2).

The morphology of 5-day muscle grafts is shown in Figure 4. To reveal the structure within the tissue, the graft was progressively fractured with a razor blade (Fig. 4A). In the PL-treated graft (Fig. 4A, B, D), the
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Figure 5. Morphology of a PL-treated muscle graft maintained for 12 days on the CAM as revealed by scanning electron microscopy. A. Low magnification view of the fractured muscle graft. Arrow indicates site with cartilaginous phenotype. B, C. Higher magnifications of the site indicated in (A) before the deep fracture, showing numerous round cells (solid arrows) embedded in abundant extracellular matrix (open arrows) of a morphological consistency similar to that in cartilage (see Fig. 3C). Fibrous material, which was prevalent throughout the graft, was seen surrounding the cartilaginous site (B). D. Higher magnification of the same site as indicated in A, showing the continuous cartilage-like morphology of abundant extracellular matrix. Bar denotes 100 µm in A and 10 µm in B, C, and D.

presence of fibrillar material was apparent; however, the fibers were generally disorganized (Fig. 4B), as compared to those in the muscle (see Fig. 3A, B) from which the explants were derived. Similar, disorganized fibers were also seen in control grafts (Fig. 4C). Occasionally, areas containing cells of a round morphology could be seen in the core of the PL-treated grafts (Fig. 4D), corresponding perhaps to the large cells seen in Fig. 2E. In general, the scanning electron microscopy morphology of the grafts was in agreement with the histology revealed by light microscopy (see Fig. 2).

As stated above, grafts maintained for a longer period, i.e., 12 days, on the CAM and treated with 6 injections of PL appeared to be much more condensed than the 5-day grafts, with the occasional presence of a hard, central core. Scanning electron microscopy again revealed collapsed and disorganized fibrous tissue (not shown), as in the 5-day muscle grafts. In one instance, a dramatic change in phenotype was observed (Fig. 5). An internal region of the graft (Fig. 5A) was found to contain a large number of round cells embedded in abundant extracellular matrix (Fig. 5B-D). This morphology was highly analogous to that of a typical cartilage (see Fig. 3C, D).

Biosynthesis of Cartilage Matrix

The extent of biosynthesis of sulfated glycosaminoglycans, a hallmark of the cartilage matrix, was compared between the control and PL-treated grafts based on the metabolic incorporation of [35S]sulfate by the grafts in organ culture. The data in Fig. 6 showed that the PL-treated grafts showed a significantly greater level (2- to 3-fold) of [35S]sulfate incorporation than the controls, consistent with the presence of a larger amount of cartilage-like matrix in the former. Biochemical characterization of collagen type biosynthesis was carried out by
Figure 6. [$^{35}$S]Sulfate incorporation by muscle grafts. Biosynthesis of sulfated glycosaminoglycans in the muscle grafts was estimated by organ culture and [$^{35}$S]sulfate metabolic labelling as described in Materials and Methods. Control and PL-treated grafts were compared based on the level of [$^{35}$S]sulfate incorporation per mg dry weight of the tissue, and expressed as a percentage of the control value. (A) and (B) represented the results of two separate, 12-day graft experiments. Three to four grafts in each experiment were individually organ cultured and analyzed. The values are mean ± standard error (P < 0.05 between control and PL-treated for both experiments).

Figure 7. Characterization of collagen type synthesis by muscle grafts. Metabolically [$^{3}$H]proline-labelled grafts (control and PL-treated) were analyzed by SDS gel electrophoresis and fluorography as described in Materials and Methods. A. Fluorograph of SDS gel showing the position of collagen $\alpha_1$ and $\alpha_2$ chains in representative samples of control and PL-treated grafts. B. Densitometric quantitation of collagen $\alpha_1:\alpha_2$ ratios in control and PL-treated grafts. The values represent the mean ± standard error of 5 grafts in each group (P < 0.05 between control and PL-treated).

means of SDS gel electrophoresis of the extracts of muscle grafts metabolically labelled with [$^{3}$H]proline in organ culture (Fig. 7). As shown in Fig. 7A, two prominent radiolabelled protein bands, corresponding to collagen $\alpha_1$ and $\alpha_2$ chains, were seen in both control and PL-treated samples. Densitometric tracing and quantitation of the relative intensity of the two a chains showed that in control grafts, the $\alpha_1:\alpha_2$ ratio was 2.1, whereas in the PL-treated samples, the ratio was 3.5 (Fig. 7B). Since type II collagen, the cartilage-specific collagen type, is a homotrimer composed of $[\alpha_1]_3$ (Mayne, 1984), the higher $\alpha_1:\alpha_2$ ratio of the PL-treated grafts was indicative of the presence of cartilage.

Discussion

This study represents the first experimental test of whether the polycation, PL, a potent chondrogenesis-stimulating agent in vitro (San Antonio and Tuan, 1986, 1987), is also capable of inducing cartilage formation in ectopic tissue sites in vivo. Muscle explants maintained as tissue grafts in vivo permit isolated, direct manipulations, and have been used here as the experimental system to test the effect of PL in vivo. The criteria used here to evaluate the appearance of cartilage include alcian blue staining, general histology with H-E staining, morphology based on scanning electron microscopy, and biochemical characterization of matrix biosynthesis. The results presented here clearly demonstrate that PL treatment of the explants has enhanced cartilage formation as evidenced by the increased abundance and frequency of alcian blue staining, biosynthesis of cartilage-specific extracellular matrix, and, in one instance, the appearance of typical cartilage morphology within a PL-treated graft.

The PL treatment used here is in the form of multiple injections of a PL solution in physiological saline. High-molecular-weight, 398 KD PL is used since the chondrogenesis-stimulating effect of PL is stringently dependent on its molecular size (San Antonio and Tuan,
The amount of PL introduced into the grafts is calculated based on the optimal, effective concentration in vitro (5 µg/ml) (San Antonio and Tuan, 1986) and the approximate tissue volume (~0.125 ml) of the explants. This dose is non-toxic since necrotic deterioration of the grafts, an inherent complication of tissue grafting, takes place in 33% of the explants and appears to be random and bears no relation to PL injection. For most of this study, we have chosen to examine the explants after at least 5 days of grafting, since the chondrogenesis-stimulating activity of PL has been found to be maximal after 3 or more days of application in vitro (San Antonio and Tuan, 1986). It should be pointed out that many of the explants (56%) were unable to be harvested at the end of the experimental period, because they slipped over the edge of the CAM of the SL embryo, i.e., lodged between the CAM and the plastic wrap, thus becoming engulfed by the expanding CAM or pushed into the albumen. Experimentally, this is an important aspect to take into consideration, because sample numbers are essentially reduced by half. On the other hand, the viability of the explant itself is probably dependent on the extent of tissue and vascular integration between the graft and the host CAM. Interestingly, when the explant is maintained for an extended period, i.e., 12 days, it becomes considerably more condensed and contracted. Tissue viability does not seem to be further compromised; however, since only viable explants are transferred on day-5 of grafting to a new host CAM, it is likely that such explants have already been "adapted" to grafting. Finally, the additional PL injections also do not appear to be toxic, suggesting that the dose used here is biologically acceptable or tolerable in vivo.

The indication of enhanced cartilage formation in the PL-treated muscle grafts is consistent with the chondrogenesis-stimulating action of PL in vitro, using embryonic limb bud mesenchymal cells (San Antonio and Tuan, 1986). However, the effect reported here is mostly a statistical one since, in most cases, the histology and morphology of the PL-treated muscle grafts fail to show cells with a definitive chondrocyte phenotype (Figs. 2 and 4). The one exception was found in a 12-day graft, where a dramatic, cartilage-like phenotype was seen (Fig. 5), showing round cells embedded in abundant extracellular matrix. Thus, it may be argued that the majority of the alcan blue positive cells are only "chondroprogenitor" cells, i.e., they are on their way to becoming fully mature chondrocytes and consequently do not display the rounded morphology characteristic of the latter, which results in part from the expansive extracellular matrix produced by mature cartilage. However, it is noteworthy that these "chondroprogenitor" cells are capable of synthesizing increasing amounts of sulfated glycosaminoglycans (consistent with alcan blue staining) (Fig. 6) as well as the cartilage-specific collagen type II (Fig. 7). Since our initial observations were made with 5-day grafts, it was thought that a longer period of PL treatment could conceivably result in the appearance of mature chondrocytes. As described above, although the 12-day grafts generally appeared more condensed, their histology did not change significantly from that of day-5 grafts; however, in one instance, genuine cartilage phenotype was observed. A feasible approach to further evaluate the chondrogenic potential of the cells of the PL-treated grafts is to enzymatically dissociate the grafts into single cells at the end of the experimental period, and to examine subsequently the differentiation of these cells in vitro, using criteria for chondrogenesis similar to those used for embryonic limb mesenchymal cells in culture (Ahrens, et al., 1977; Evans and Tuan, 1988; Gawande and Tuan, 1990; San Antonio and Tuan, 1986; Solursh, 1984a, b; Solursh, et al., 1978).

The mechanism of cartilage induction is an area actively being studied by many research groups using a number of approaches, many of which have involved the isolation and functional characterization of cartilage-inductive factors derived from the bone matrix (e.g., Bentz, et al., 1989; Canalis, et al., 1988; Caplan and Pechak, 1987; Gawande and Tuan, 1990; Haenschka, et al., 1986; Kawamura and Urist, 1988; Hauschka, et al., 1987). Our previous studies (San Antonio and Tuan, 1986, 1987; San Antonio, et al., 1987; San Antonio, et al., manuscripts submitted) have shown that polyionic interactions involving extracellular and cell surface components are important regulators of cartilage development in vitro. Reagents that influence these interactions in vitro include PL (San Antonio and Tuan, 1986, 1987) and glycosaminoglycans (San Antonio, et al., 1987). Based on these findings, we have recently proposed a "Matrix Trapping" mechanistic model for chondrogenesis (Tuan, 1991). The central theme of this model proposes that exogenous polyions stabilize cartilage nodule formation and extracellular matrix expression by promoting the trapping of cartilage matrix components. The process is believed to proceed via the following steps:

1. Polyionic substances interact with an endogenous counter-polyionic complement molecule produced by the mesenchymal cell. Thus, PL may bind an anionic proteoglycan(s), and heparin or heparan sulfate may bind the cationic domains of the II collagen, or other matrix components.

2. The polyionic complex formed in this manner may promote or stabilize chondrogenesis by creating a pericellular "trap" to encapsulate the emerging cartilage nodule, and thus allow undisturbed assembly of the extracellular matrix, or the progression of cell-cell interactions required for cartilage matrix biosynthesis. Alternatively, the pericellular matrix may also serve to elevate the effective concentration of inductive factors, such as TGF/β (Seyedin, et al., 1987), which enhance chondrogenesis.

A corollary to this model is that it also provides a relatively simple explanation for the cell density dependence of chondrogenesis in vitro (Ahrens, et al., 1977). When limb mesenchymal cells are cultured at greater than confluent densities, the cartilage matrix
secreted by some of the chondroblasts may be trapped within the surrounding cells, which provide the partitioned environment for matrix assembly and the emergence of the nodule. Similarly, cells cultured clonally within an artificial matrix of collagen gel or agarose (Solursh, et al., 1982) would also experience such matrix trapping, and are thus capable for forming cartilaginous colonies. On the other hand, cells in cultures seeded at less than confluent densities may also produce cartilage matrix, but in this case the matrix is not stabilized in its assembly, and the nodule subsequently regresses or is never formed. Indeed, a cartilage nodule may simply be a manifestation of the undisturbed, cooperative assembly of cartilage matrix by a group of chondroblasts. Results from this study, using tissue grafts, may simply be a manifestation of the undisturbed, cooperative assembly of cartilage matrix by a group of chondroblasts. Results from this study, using tissue grafts, strongly imply that these interactions are most likely to be also functionally important in vivo. Perhaps the PL treatment of the muscle grafts causes agglutination of a significant number of stem cells within the graft; subsequent matrix trapping then leads to the appearance of cartilage phenotype. This hypothetical model of the mechanism of PL action remains to be tested. On the other hand, it should be of great interest to evaluate the efficacy of PL treatment on chondrogenesis or cartilage growth by direct injection or delivery of PL into intact tissue sites in vivo. Such knowledge should be invaluable in eventually identifying agents and treatment procedures that are capable of reproducibly stimulating cartilage production at specific sites. The results presented here suggest that PL, a chemically defined polyionic polymer, may serve to facilitate such applications.

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References


Discussion with Reviewers

B.K. Hall: What is the evidence that muscle contains stem cells? Are the alican blue positive cells after PL treatment derived from dedifferentiated myoblasts, fibroblasts, or resident stem cells?

Authors: There is no direct evidence for the presence of stem cells in muscle, although the fact that various inductive substances derived from the bone matrix are able to elicit chondrogenic and osteogenic responses when implanted in muscle, even in X-irradiated animals, strongly suggests the presence of pluripotent cells in the local environment. The exact cellular source for the cells producing an alican blue positive matrix is not known at present.

B.K. Hall: Does seepage of PL from one injected graft to a neighboring uninjected graft compromise the analysis of the localized effect of PL?

Authors: The seepage results from the fairly large injection volume or using a colligative carrier, such as collagen.

B.K. Hall: Since morphologically distinct chondroblasts are seen in only one graft, are the results consistent with...
chondrogenesis or simply initiation of polysaccharide synthesis?

**Authors:** The morphological results presented here certainly do not distinguish between these two possibilities, in particular since the SEM analysis may have overlooked certain regions of the tissue graft. However, the apparent synthesis of collagen type II, coupled with increased $[^{35}S]$ sulfate incorporation, would favor genuine chondrogenesis.

M. Solursh: Is there other biochemical evidence that collagen type II is indeed synthesized by the PL-treated grafts, since the increased $\alpha_1:\alpha_2$ ratio may be due to either the synthesis of $\alpha_1$ trimer or the degradation of $\alpha_2$ chains?

**Authors:** We agree that definitive confirmation of collagen type II production requires additional biochemical and molecular characterization. Also, whether $\alpha_2$ is lost or not remains to be determined, although since the tissue was extracted in toto and not differentially fractionated for SDS PAGE analysis, the loss of $\alpha_2$ during extraction should be minimal, or at least equivalent between the control and PL-treated samples. The data are therefore consistent only with an apparent expression of collagen type II in the PL-treated grafts.

A. Ornoff: Is it possible to directly compare the SEM and LM findings on the same specimen?

**Authors:** Although we agree with the reviewer that it would be ideal to actually compare the LM and SEM findings on the same specimen, it is however practically impossible since the cartilage-like regions are isolated and occur randomly among the specimens. It is therefore not feasible to predict *a priori* which specimen is going to display "interesting" morphology. In fact, we have dissected some specimens into two halves and, more often than not, have seen cartilage-like regions in one half and not the other. For example, the LM histology of one particular graft with a cartilage-like area is shown in Fig. 2G, H.

C.D. Little: To what cells do the "round cells" in Fig. 4 correspond?

**Authors:** The "round cells" shown in Figure 4 are likely to correspond to those found in the histology sections in Figure 2 and localized in the areas surrounded by alcian blue-positive matrix. Although Figure 4 does not reveal dramatic morphological changes as shown in Figure 5, this is exactly the point, i.e., most grafts indeed only display this type of morphology when examined as described here. These areas most likely correspond to the alcian blue-positive zones by histology. Also, according to our observation, the large, round cells are more frequently seen in the PL-treated grafts. Whether similar looking cells, sometimes found in control grafts, are actually equivalent to those in PL-treated grafts is not known.