Arachidonate Metabolism in Cultured Fibroblasts Derived From Normal and Infarcted Canine Heart

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Metabolites of arachidonic acid (eicosanoids) may have an important role in the healing process after myocardial infarction. We examined the ability of cardiac fibroblasts from normal and from healing infarcted ventricle to metabolize arachidonate. We induced myocardial infarction in dogs and then allowed them to recover for 1 week, at which time they were killed, and the heart was removed. Fibroblasts were harvested from normal and from the healing, infarcted areas of the left ventricle. The cells from each source were morphologically indistinguishable. There were 347 ±102-fold more fibroblasts cultured from the infarcted area than from the normal area. Interestingly, the infarct-derived cells had a slower doubling time (37.4±3.7 hours) than the normal cells (22.0±3.6 hours). The uptake of exogenous arachidonate and its distribution in complex lipids was the same in the cells from each area. When stimulated with the calcium ionophore, free exogenous arachidonate, bradykinin, or histamine the cells produced prostaglandin E\textsubscript{2} and prostaglandin I\textsubscript{2}. In each case the infarct-derived cells produced from twofold to fivefold more prostaglandin than the normal cells. We also found that prostaglandin synthesis was highly dependent on the growth state of the cells with a marked decrease a confluence. Finally, in experiments designed to mimic the early state of infarction, we confirmed that isolated cardiac myocytes release arachidonate and showed that normal fibroblasts can incorporate it. The production of eicosanoids by cardiac fibroblasts may be substantial during the healing of myocardial infarction due to their dramatic proliferation and the increased prostaglandin production per cell. (Circulation Research 1989;65:671–683)
esis for these experiments was that fibroblasts from healing myocardium would exhibit altered arachidonate metabolism. This idea was based on results in synovial cells, which exhibit increased prostaglandin synthesis when derived from an inflamed joint as compared with a normal one. The alteration in prostaglandin synthesis is probably due to the action of interleukin-1. Exposure to monocyte secretory products increases the production of PGE₂ by skin fibroblasts, and the proliferation of fibroblasts may explain a portion of the increased eicosanoid production in hydropnephrotic kidney.

An increased production of prostaglandins by fibroblasts during the healing of a myocardial infarction could have widespread implications. For example, free arachidonate, PGI₂, and prostaglandin F₂α have been shown to increase the rate of protein turnover in cardiac muscle, and endogenous prostaglandins have been implicated as facilitating the toxic effects of cardiac glycosides. The amount of PGI₂ produced by acutely ischemic myocardium has been shown to be inversely correlated with the occurrence of arrhythmias, and prostaglandin F₂α has been shown to affect both electrical and mechanical function of cardiac tissue. Also, inhibitors of cyclooxygenase have been shown to block the arrhythmogenic properties of acetylstrophanthidin. The effects of PGI₂ on vascular resistance and of both PGI₂ and PGE₂ on the inflammatory response may be important during myocardial healing. Given the diversity of their effects, one could imagine both favorable and detrimental results from a marked increase in prostaglandin production.

In the experiments reported here, we show that cultures of fibroblasts can be established from both normal and healing, infarcted myocardium. There are many more of such cells in the infarcted region, and their growth rates in culture are different from the rates of cells from normal regions. Fibroblasts from both sources rapidly incorporate arachidonic acid supplied exogenously or from dying isolated myocytes. The fibroblasts from each source produce PGI₂ and PGE₂ when appropriately stimulated, and the infant-derived cells have a twofold increase in PGI₂ production as compared with cells derived from normal ventricle. The marked increase in the number of fibroblasts in the area of a healing myocardial infarction, their increased production of a potent eicosanoid, and their ability to use arachidonate released from dying myocytes all suggest that these processes may influence healing after myocardial infarction.

Materials and Methods

The radiolabeled reagents were purchased from NEN, Wilmington, Delaware: [³H]6-ketoprostaglandin F₁α ([³H]6-ketoPGF₁α, 120–180 Ci/mmol), [³H]PGE₂ (100–200 Ci/mmol), [¹²⁵]I 3’,5’-cyclic AMP (2,200 Ci/mmol), and [³H]arachidonic acid (60–100 Ci/mmol). Medium 199, Costar flasks, and petri dishes were purchased from Hazleton, Lenexa, Kansas. Antiserum to PGE₂ was from Advanced Magnetics, Cambridge, Massachusetts, and antiserum to 6-ketoPGF₁α was a gift from Dr. Nancy Baenziger, Washington University, St. Louis, Missouri. Prostaglandin standards and cyclic AMP were from Sigma Chemical, St. Louis, Missouri. Joklik medium, collagenase (200 units/mg), and hyaluronidase were purchased from GIBCO Laboratories, Grand Island, New York. The antiserum to cyclic AMP was purchased from Miles Laboratories, Elkhart, Indiana.

Cell Culture

Induction of myocardial infarction. Mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg), and were maintained on a ventilator. Under aseptic conditions, a thoracotomy was performed through the fourth intercostal space. The pericardium was opened, and the left anterior descending coronary artery or two branches of the left anterior descending coronary artery were isolated and ligated with suture. The incision then was closed, and the dog was allowed to recover. Antibiotics were given for 3 days postoperatively, and analgesics were administered as needed. These experiments were performed with the approval of the Animal Use Committee of the University of Utah.

Harvest of myocardial fibroblasts. On the seventeenth day after infarction, the heart was excised aseptically from the anesthetized dog. All subsequent procedures were performed in a laminar flow hood by aseptic technique. The tissue was rinsed with ice-cold Joklik medium, and both atria and the right ventricle were removed. The left ventricle was placed on a dissecting dish and opened with the endocardial surface superior, and the endocardium was removed. The procedure for producing an infarction typically resulted in an obvious injury of approximately 2×3 cm on the endocardial surface. The endocardial surface was necrotic and friable, whereas the epicardial surface usually showed only mild mottling. Portions of the ventricle from the healing infarction and from normal areas were selected for culture and were processed separately. The tissue was placed in Joklik medium and minced with surgical scissors into pieces of approximately 2 mm³. The medium was removed, and the wet weight of the tissue was determined. The tissue then was incubated in Joklik medium that contained collagenase (200 units/ml) and trypsin (200 units/ml) for five periods of 20–40 minutes each at 37° C. At the end of the period, the supernatant was removed from the sedimented tissue and was centrifuged (250g for 5 minutes). The cellular pellet obtained was resuspended in growth medium (Medium 199 with 10% fetal bovine serum, penicillin [100 units/ml], and streptomycin [100 µg/ml]) and plated in T-75 flasks. The cells from each incubation period were pro-
cessed and plated in this manner. The rationale for multiple incubations of 20–40 minutes rather than a single longer incubation was that the shorter exposure of dissociated cells to the proteases would result in healthier cells. In a typical experiment, the first two incubations yielded far fewer fibroblasts than the third through fifth incubations. Two hours after the initial plating, the medium was removed, and fresh medium was added. This step serves to remove cellular debris. After 24 hours, all flasks were treated with trypsin-EDTA, and the cells were pooled and inoculated into dishes (28.3 cm²) unless otherwise indicated. It was at this time that the number of cultured cells per gram of wet tissue was determined. This subculture was performed to remove cellular debris so that an accurate cell count could be performed and the fibroblasts could be plated at a density that was conducive to growth.

The cells were identified as fibroblasts (or an equivalent mesenchymal cell) by their appearance on phase-contrast microscopy. Macrophages were not present in the cultures as assessed by microscopy. Also, the morphology didn’t change during serial passage, thereby excluding substantial contamination.

**Growth of fibroblasts.** The cells were maintained at 37°C in a humidified atmosphere composed of 5% CO₂-95% air. Approximately 75% of the medium was removed and replaced twice per week. At confluence the fibroblasts were treated with trypsin-EDTA and passed at the same density as the initial plating. The plating efficiency was approximately 20% for normal cells and 28% for cells derived from an infant that was plated at 100 cells/25 cm².

**Cardiac Myocyte Isolation**

The isolation was a modification of the procedure described by Isenberg and Klockner.¹⁸ Thoracotomy was performed via the fourth intercostal space of an anesthetized normal dog, and the heart was removed. The heart was perfused by gravity flow with 1 l ice-cold Ca²⁺-free oxygen-saturated buffer until the tissue paled. Strips of the right ventricle were removed and minced into pieces of approximately 4 mm³. The tissue was allowed to equilibrate in the same buffer at 37°C. All solutions used in the isolation procedure were saturated with oxygen and maintained at 37°C in a shaking water bath. The tissue suspension was bubbled with oxygen, and the buffer was changed every 5 minutes for 30 minutes. The tissue then was exposed to the Ca²⁺-free buffer containing collagenase (1 mg/ml) and hyaluronidase (1 mg/ml) for five periods of 30 minutes each. The supernatants from the first three periods contained cellular debris and were discarded. The supernatants from the final two incubations were pooled to yield the isolated myocytes. The cells were assessed at this stage by light microscopy, and we found two populations of myocytes: cells with a rectangular shape and distinct striations and globular cells without striations. In a typical experiment (n=10), approximately 50% were rectangular. The rectangular cells excluded trypan blue whereas most of the globular cells did not. This observation is consistent with that of Isenberg and Klockner.¹⁸ These cells were centrifuged at approximately 50g for 4 minutes to form a soft pellet. Most of the supernatant was aspirated and replaced with a Kraftbrühe (KB) buffer for 60 minutes in a final volume of 100 ml. KB buffer (Isenberg and Klockner) contained (mM) KCl 85, K₂HPO₄ 30, MgSO₄ 5, Na₂ATP 5, pyruvic acid 5, β-OH-butyric acid 5, creatine 5, taurine 20, glucose 20, EGTA 1, and (g/l) polyvinyl-pyrrolidone 50 at pH 7.2. In experiments using radiolabeled myocytes, 50 μCi [³H]arachidonic acid (final concentration, 5.7 nM) was added for the final half hour of this incubation. The myocytes were washed, and unincorporated [³H]arachidonic acid was removed by resuspending them twice in normal Tyrode’s buffer that contained 0.5 mg/ml essentially fatty acid–free bovine serum albumin.

**Arachidonic Acid Metabolism**

In all experiments with exogenous arachidonic acid, either labeled or unlabeled, controls that omitted a source of enzymatic conversion were included (e.g., incubations in buffer alone) under otherwise identical conditions. The arachidonic acid was monitored by thin-layer chromatography (TLC) routinely (every few weeks) for degradation and was repurified by TLC or high-performance liquid chromatography (HPLC) if more than 1–2% contamination was present.

**Radiolabeled studies.** Fibroblasts were labeled by incubation for 3 hours with 1 μCi [³H]arachidonic acid and 1 μM unlabeled arachidonic acid in Medium 199 that contained 0.5 mg/ml essentially fatty acid–free bovine serum albumin. The fibroblasts incorporated 70–90% of the radiolabel during this time. Myocytes were labeled for 30 minutes as indicated above. The short labeling period was chosen to preserve the cell viability; however, only 26% of the [³H]arachidonic acid was incorporated during this time (n=9).

**Phospholipid extraction.** The phospholipids from the fibroblasts or myocytes were extracted by the technique of Bligh and Dyer.¹⁹ The phospholipids were separated by TLC on silica plates in CHCl₃:MeOH:CH₃CHOH:H₂O (25:15:4:2). The fractions from a TLC lane were scraped, and the radioactivity was determined by liquid scintillation spectroscopy. The lipids were identified by comparison of their Rf's with those of authentic standards. In some experiments, we separated phospholipids by HPLC.²⁰

**Arachidonic acid metabolism.** The metabolism of arachidonic acid was determined by prelabeling the cells with [³H]arachidonic acid, followed by stimulation with an agonist. The supernatant was removed, acidified, and extracted twice with 5 vol ethyl acetate. The prostaglandins were separated by TLC²¹ and were identified by comparison with...
authentic standards. In some experiments, arachidonic acid metabolites were measured by HPLC. Briefly, we used an octadecylsilyl column (4.6×150 mm, 5 μm Ultrasphere, Beckman, San Ramon, California) and a mobile phase of acetonitrile in aqueous H₃PO₄ (pH, 3.5) at 2 ml/min. The initial composition was 26% acetonitrile, with step increases at 34 minutes and 70 minutes to 50% and 100%, respectively. The radiolabeled experimental samples were identified by comparing their elution volumes with those of authentic standards. The radioactivity of the fractions was determined by liquid scintillation spectroscopy.

Quantitation of PGE₂ and 6-KetoPGF₁α. PGE₂ was quantified by radioimmunoassay according to the supplier's instructions (Advanced Magnetics). The radioimmunoassay of 6-ketoPGF₁α has been described previously. Briefly, the cells were exposed to media with or without an agonist for 2 hours. Each assay tube contained 0–450 μl of the supernatant, antisera, and the radioisotope in a final volume of 525 μl. After a 16–24-hour incubation, the free ligand was precipitated with activated charcoal. The PGE₂ assay was linear from 30 to 250 pg PGE₂ in the assay, and the 6-ketoPGF₁α assay was linear between 25 and 250 pg 6-ketoPGF₁α.

Miscellaneous

Transmission electron microscopy was performed at the Electron Microscopy Center, Salt Lake City Veterans Administration Medical Center, Department of Pathology as described. Radioimmunoassay of cyclic AMP was performed as described previously. Gas-liquid chromatography of the methyl esters of fatty acids was performed as described. The data were analyzed with a paired t test.

**FIGURE 1.** Electron microscopic examination of normal and infarcted myocardium. Myocardial infarction was induced in anesthetized dogs by ligating the anterior descending coronary artery. The animals were allowed to recover, and 1 week later they were killed and the heart was removed. Portions of the heart from normal areas and from areas that were healing after the infarction were removed and processed for electron microscopy. Panel A: A photomicrograph from a normal area of the ventricle (magnification, ×11,225). Panel B: A photomicrograph (magnification, ×14,030) from an infarcted area. Note the marked edema, the membrane disruption, and the loss of organization. Panel C: A photomicrograph from a healing infarcted area. This field shows the presence of three cells between myofibrils that have the typical morphological appearance of a fibroblast. Each of the panels shown is representative of many fields studied from several blocks in this animal. These results were confirmed in two other experiments in which tissue samples were taken from normal and infarcted areas. The proliferation of the fibroblasts was a typical finding in the healing areas.
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Arachidonate Metabolism in Cardiac Fibroblasts

Results

Characteristics of Fibroblasts in Cell Culture

In each experiment, fibroblasts were derived from myocardium that had been allowed to recover from an infarct for 7 days. Fibroblasts were obtained from a normal area of the left ventricle in the same heart to serve as the control population. In preliminary experiments, with cells from entirely normal hearts, we found that their appearance and prostaglandin production were the same as the appearance and prostaglandin production of cells derived from a normal area of an infarcted heart (data not shown). The area affected by the infarct was easily distinguished from normal myocardium by light-colored patches of tissue at the macroscopic level. In the infarcted areas, necrotic myocytes and a proliferation of fibroblasts were demonstrated by transmission electron microscopy (Figure 1). The number of fibroblasts derived from infarcted tissue per gram of wet weight was 347±102-fold greater than the number of fibroblasts derived from normal tissue (mean±SEM; n=10; the range was from 35- to 1,000-fold). Morphologically, fibroblasts derived from either area of myocardium were similar, with the characteristic spindle shape typical of normal fibroblasts (Figure 2). Clark et al27 have noted a change in the type of collagen produced by cells after activation. This may correlate with our observation that the fibroblasts derived from infarcted tissue required a longer period of exposure to trypsin-EDTA before the cells detached (not shown). Fibroblasts derived from normal myocardium grew faster than the fibroblasts derived from infarcted tissue (Figure 3). The doubling time, as determined by cell counts, during exponential growth was 22.0±3.6 hours (mean±SEM) for normal fibroblasts whereas it was 37.4±3.7 hours for infarct-derived fibroblasts (p=0.02; n=6). This observation was confirmed by measuring the increase in protein content (n=13) and the incorporation of [3H]thymidine into DNA in the two populations (n=2) (data not shown). Examination of the growth rate, based on the protein content in three different experiments, indicated that this difference was maintained for at least four passages in culture.

Figure 2. Microscopic appearance of cultured myocardial fibroblasts. Fibroblasts were obtained from a normal area of canine heart as described in “Materials and Methods.” The appearance by inverted phase-contrast microscopy (magnification, ×400) is shown. The appearance of fibroblasts derived from normal ventricle and healing, infarcted ventricle was identical (not shown). The appearance shown in these photomicrographs was typical of that of fibroblasts cultured from 10 dogs. This same appearance was seen through at least four passages of fibroblasts. Panel A: Cells 3 days after plating; Panel B: Cells just before confluence.

We considered the possibility that an increased production of PGI2 or PGE2 by the infarct-derived cells led to an increased concentration of cyclic AMP in the cell and thereby decreased their rate of growth as compared with normal cells. In two experiments, we treated the infarct-derived fibroblasts with aspirin (100 μM) or indomethacin (1 μM), inhibitors of cyclooxygenase, and compared their rate of growth with infarct-derived cells without such treatment. The inhibitors were present throughout a growth-rate experiment like that shown (Figure 3) and were shown in control experiments to block PGE2 and PGI2 synthesis by over 90%. We found that neither treatment altered the growth of either the normal or infarct-derived cells (data not shown). Cyclic AMP has been implicated in the regulation of cell growth, so we examined these cells for differences in cyclic AMP production. The fibroblasts produced the same amount of cyclic AMP in the basal state (1.0 nmol/mg protein for normal cells, 1.2 nmol/mg protein for infarct-derived cells) and when stimulated with forskolin (11.5 nmol/mg protein for normal cells, 10.0 nmol/mg protein for infarct-derived cells). This was confirmed in a second experiment in which it was also shown that the proportion of intracellular versus secreted cyclic AMP and the total amount of cyclic AMP were the same in the two populations after forskolin (normal, 216 pmol/plate intracellular and 28 pmol secreted; infarct, 232 pmol/plate intracellular and 42 pmol secreted).

Arachidonic Acid Release and Metabolism by Fibroblasts

A primary goal of this investigation was to study the metabolism of arachidonic acid in these two populations of cells. We examined arachidonic acid metabolism by TLC (not shown) and HPLC (Figure 4) of supernatants from cells prelabeled with [3H]arachidonic acid. We found that these cells released free [3H]arachidonic acid and metabolites that had retention volumes identical to 6-ketoPGF1α (the stable metabolite of PGI2), PGE2, and hydroxy fatty acids when the cells were pretreated with the calcium ionophore, A23187 (Figure 4). Cells derived from normal and infarcted areas exhibited the same spectrum of products in four experiments from separate isolates.

Quantitation of PGE2 and 6-ketoPGF1α produced from endogenous arachidonate revealed considerable variability, which was partially dependent on the length of time between plating and the stimulation of the cells. As had been noted in other cell types,28 these fibroblasts produced more prostaglandins (per milligram protein) during exponential growth than at confluence (Figure 5). In view of this effect of the state of cell growth on arachidonic acid metabolism, all experiments that compared normal fibroblasts with infarct-derived fibroblasts were performed simultaneously on both cell populations, which had been plated at 105 cells/dish (28.3 cm2)
and grown under identical conditions. The protein content was measured in each plate, and prostaglandin production was expressed as related to this value. In the absence of a stimulus, only minimal production of any eicosanoid was observed during a 2-hour incubation, and stimulation with either A23187 or arachidonic acid caused a significant increase in both PGE$_2$ and 6-ketoPGF$_{1\alpha}$ synthesis when quantified by radioimmunoassay. The production of 6-ketoPGF$_{1\alpha}$ by infarct-derived fibroblasts was twofold to fivefold greater than that produced by normal fibroblasts. As mentioned above, there was considerable variation, yet this difference between normal and infarct-derived fibroblasts was always apparent between matched experimental pairs (eight experiments). When analyzed statistically (Table 1), there was a significant difference on days 3–9 after plating, that is, during the time that they were subconfluent. At confluence this difference had disappeared. During the 2-hour pretreatment (i.e., in the absence of an agonist), fibroblasts did not spontaneously synthesize measurable amounts of eicosanoids. However, when the growth medium from a 24-hour period was tested by radioimmunoassay for PGE$_2$ and 6-ketoPGF$_{1\alpha}$, significant amounts were detected. As in the stimulated samples, the infarct-derived fibroblasts produced more 6-ketoPGF$_{1\alpha}$ than normal fibroblasts (data not shown). The increased production of prostaglandins by infarct-derived cells could have been due to an increased release of free arachidonate from its esterified position in phospholipids or to increased cyclooxygenase activity. We examined this by measuring the response to the calcium ionophore A23187, which stimulates release, and the response to saturating concentrations of exogenous arachidonic acid, which is directly used by cyclooxygenase activity. 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Various possible agonists that induce prostaglandin production in other cells and might be relevant in this setting were examined in confluent cells. These included bradykinin, histamine, thrombin, epinephrine, isoproterenol, forskolin, and streptokinase. Of these agents only bradykinin and histamine stimulated production of 6-ketoPGF$_{1\alpha}$ above control (i.e., buffer only), and as shown in Table 2, the difference between normal and infarct cells was again observed.

![Graph showing growth characteristics of fibroblasts derived from normal canine myocardium or healing, infarcted myocardium. Fibroblasts were obtained from either normal or infarcted areas of the hearts as described in "Materials and Methods." After 24 hours in culture, the debris was washed away carefully, and the fibroblasts were harvested with trypsin-EDTA. From each pool of cells, $10^5$ were plated onto each dish and then cultured in the same manner. At the time shown, plates were taken for removal of cells, and their number was determined in a Coulter counter. Note that the ordinate is a log scale. The results shown are from a single experiment with the points performed in duplicate and are representative of the results obtained in five additional experiments with cells from different animals. Closed circles, fibroblasts from a normal area of myocardium; open circles, fibroblasts derived from an infarcted area.](http://circres.ahajournals.org/doi/abs/10.1161/01.CIR.65.3.678)
Figure 4. Graph showing arachidonic acid (AA) metabolism by cultured fibroblasts. Fibroblasts obtained from an infarcted area were labeled with [3H]arachidonic acid before stimulation with the agonist A23187 for 2 hours (10 μM). The metabolites were extracted as described in “Materials and Methods” and were separated by high-performance liquid chromatography with a Beckman ODS silica column with a mobile phase of aqueous phosphoric acid (pH 3.5) and acetonitrile at 2 ml/min (“Materials and Methods”). The radioactivity in 1-minute fractions was quantitated by liquid scintillation spectroscopy. This represents a single experiment that was representative of the results obtained in three independent experiments. The pattern of metabolites was the same in cells derived from infarcted areas and normal areas (not shown). Control plates, that is, [3H]arachidonate in buffer but without cells, and labeled cells without agonist were examined in parallel. Little metabolite was found in either case, and the results shown represent the increase per fraction over the no-cell control.

PGF\(_{1α}\), prostaglandin F\(_{1α}\); PGE\(_{2}\), prostaglandin E\(_{2}\); AA, arachidonic acid.

Fibroblast Incorporation of Arachidonic Acid

In view of our finding of increased prostaglandin product in infarct-derived fibroblast, we examined other aspects of the cells' handling of arachidonate to determine whether the change was selective. We first measured their uptake of arachidonic acid and its incorporation into complex lipids. The fibroblasts readily incorporated 70–90% of arachidonic acid (1 μM) over a 60-minute incubation, as determined by the presence of a trace amount of [3H]arachidonic acid (Figure 6A). The fibroblasts from either source incorporated arachidonic acid into complex lipids in a similar fashion. The total uptake was 70,030±20,250 cpm/plate in normal cells and 62,470±17,800 cpm/plate in infarct-derived cells (mean±SD, n=3 experiments, each performed in duplicate). The normal cells had 23.3±4.3% of the [3H]arachidonate in neutral lipids, while the infarct-derived cells had 21.0±3.6% (n=4). The distribution of the newly incorporated arachidonate among phospholipids was almost identical in each population of cells, as shown in Figure 6B (three experiments). In a subsequent experiment, we separated the extracted phospholipids by HPLC and found an identical distribution. This procedure separates phosphatidylinositol (PI) from phosphatidylserine (PS), which allowed us to determine that virtually all of the label in the PI+PS fraction (from TLC, Figure 6B) actually was in PI (in normal cells PI accounted for 92.5% of the PI+PS; in infarcted cells, 95%). Also, there was little difference in the endogenous fatty acid composition (as mole percentage) between the two cell populations as determined by gas

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<th>Prostaglandin I(<em>2) production (ng of 6-ketoprostaglandin F(</em>{1α}) per mg of protein)</th>
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Confluent fibroblasts derived from normal or infarcted, healing myocardium were incubated with histamine (10 μM), bradykinin (1 μM), or buffer alone. After 2 hours the supernatant was removed and assayed for 6-ketoprostaglandin F\(_{1α}\). The values shown represent the increment of agonist-stimulated cells over buffer control (p<0.05 in each case). These results are the means of three experiments conducted in duplicate. The differences between normal and infarct-derived fibroblasts did not reach statistical significance although the trend was the same as with exogenous arachidonate or A23187 as agonists.
Growth medium was exchanged for medium containing arachidonic acid (10 μM) on various days after plating (initial density, 10⁶ cells/plate). After 2 hours in the presence of the agonist, supernatant was removed. The prostaglandin I₂ (PGI₂, determined as 6-ketoPGF₁₀α, panel A) and the prostaglandin E₂ (PGE₂, panel B) content were determined by radioimmunoassay ("Materials and Methods"). Identical incubations were performed in the absence of cells as controls, and the results were subtracted from those shown. The results shown are the means of points from four to six experiments. * Statistically significant differences between normal and infarct-derived fibroblasts. Similar results were obtained with A23187 stimulation.

**Release of Arachidonic Acid by Cardiac Myocyte and Its Uptake by Fibroblasts**

Chien et al. demonstrated that myocytes whose energy metabolism has been disrupted with cyanide-deoxyglucose released arachidonic acid as the cellular membranes were degraded. They also showed that cardiac myocytes do not metabolize free arachidonate to eicosanoids under these conditions. Since we had shown that free arachidonate was converted to prostaglandins by cultured cardiac fibroblasts, we considered the possibility that release of arachidonate from ischemic myocytes in vivo could result in prostaglandin production. Canine cardiac myocytes were isolated and tested for their ability to take up [³H]arachidonate and incorporate it into phospholipids. In a 30-minute incubation, myocytes incorporated arachidonate (5.7 nM) into phosphatidylcholine (16.0%), phosphatidylinositol (7.1%), phosphatidylethanolamine (10.7%), and neutral lipids (64%), n=2. As a means of provoking membrane lysis and release of free arachidonate, we tested buffers containing cyanide, deoxyglucose, cyanide plus deoxyglucose, isoproterenol, or nitrogen-saturated buffer. We also examined the effect of direct transfer of myocytes from Ca²⁺-free buffer to normal Tyrode's buffer (the Ca²⁺ paradox) and the Ca²⁺ paradox with buffer containing isoproterenol. We found that the Ca²⁺ paradox in the presence of isoproterenol was the most efficient in causing the release of [³H]arachidonic acid from the myocytes, and with these conditions 45.0±11.7% was released over 2 hours (n=3). We also measured arachidonate release under the same conditions from unlabeled myocytes by gas-liquid chromatography and confirmed a time-dependent increase (n=2).

In subsequent experiments, isolated myocytes that had been prelabeled with [³H]arachidonic acid were induced to release arachidonate (as above) during coincubation with normal fibroblasts, and we demonstrated that the fibroblasts could incorporate the myocyte-derived arachidonic acid into cellular phospholipids. We compared the distribution (in fibroblasts) of newly incorporated arachidonate that had been released from myocytes with the distribution when arachidonate was provided exogenously (Figure 7). As shown, the pattern was similar, regardless of the source, although more radiolabel was incorporated from exogenous [³H]arachidonate because its specific radioactivity was much higher than that released from myocytes. We next examined whether the source of the [³H]arachidonate incorporated by ventricular fibroblasts influenced its accessibility for release. Fibroblasts were labeled by exposure to free exogenous [³H]arachidonate or to prelabeled myocytes as before. They then were washed and stimulated with the calcium ionophore A23187. We found that 8.5% of the arachidonate from labeling with free exogenous [³H]arachidonate was released and that 8.1% of the [³H]arachidonate derived from myocytes was released (mean of two independent experiments done in duplicate; expressed as percent of total cellular arachidonate released greater than buffer control).

**Discussion**

In the experiments reported here, we have demonstrated that it is possible to establish cultures of a fibroblast, or fibroblast-like cell, from canine ventricular myocardium. This work has extended the report of Ahumada et al. in that we established cultures from healing, infarcted tissue as well as...
from normal areas and have characterized the behavior of the cells more thoroughly. The cells from each source are indistinguishable by a morphological criterion, but the infarct-derived fibroblasts have a substantially slower growth rate (Figure 3). As expected, we obtained many more fibroblasts from the area of myocardium that was recovering from an infarction than from a normal area.

We also found that fibroblasts derived from the healing, infarcted myocardium produced more prostaglandins on a per cell (or per milligram of protein) basis than fibroblasts from normal myocardium. This was of interest since prostaglandins have been implicated in the healing after myocardial infarction, protein turnover in cardiac muscle, and other cardiac responses. These various effects of PGE\(_2\) and PGI\(_2\) have stimulated interest in the potential therapeutic benefits of altering their production. Our findings that the number of fibroblasts proliferates markedly during the healing phase of myocardial infarction and that the fibroblasts present during the healing response produce more PGE\(_2\) and PGI\(_2\) per cell than normal fibroblasts, suggest that the contribution of fibroblasts to the total production of these potent vasoactive and inflammatory compounds in the heart may be substantial. The increased production of prostaglandins by the infarct-derived cells appears to be due to increased cyclooxygenase activity in the cells, as has been described in fibroblasts derived from other tissues undergoing the inflammatory response, which is probably due to the presence of cytokines such as interleukin-1. We cannot exclude the possibility that the amounts of the cyclooxygenase remained the same within the infarct-derived fibroblasts as in the control cells but that the reaction catalyzed by this enzyme was influenced by other alterations in the cellular milieu. However, by analogy to other cells and tissues, this seems to be a less likely explanation. It is interesting that the increased prostaglandin production persisted in cell culture through at least four passages. This finding is similar to that of Korn, who found that the exposure of cultures of skin fibroblasts to products of mononuclear cells resulted in an increased prostaglandin synthesis and that this response persisted through many passages. He also found that his abnormal fibroblast lines grew more slowly than normal fibroblast lines but that the growth suppression did not correlate with increased prostaglandin synthesis. We observed that both the normal and infarct-derived fibroblasts made the most prostaglandins (on a per milligram of protein basis) during the phase of rapid growth, and as they approached confluence, their rate of prostaglandin synthesis declined markedly. This has also been observed by other groups including Hyman et al and appears to be due to decreased cyclooxygenase activity. This was demonstrated in our experiments by showing that the decreased production occurred.
both in response to the calcium ionophore A23187, which stimulates production by first causing release of arachidonate from phospholipid stores, or by the addition of free arachidonic acid, which serves directly as a substrate for cyclooxygenase.

These various results suggested to us that the production of prostaglandins by this type of cell might have pathophysiological importance in the heart under several circumstances. As mentioned above, the marked proliferation of fibroblasts during the healing response and the increased production of prostaglandins per cell would contribute substantial quantities of prostaglandins during the days to weeks after infarction. Many groups have been interested in the cellular sources of eicosanoids during the course of myocardial infarction. McCluskey et al. examined synthesis in microsomes prepared from an infarct at various times. They found an increase as compared with normal areas even after 3 weeks to 3 months, with 6-ketoPGF₁₀₂ and thromboxane particularly prominent. The histology at these times showed increased fibroblasts and blood vessels. Certainly the endothelial cells of the latter could be a source of PGI₂ and PGE₂. Our work suggests that fibroblasts also could be a source.

Fitzgerald et al. found that PGI₂ production (measured as a urinary metabolite) peaked within the first 24-48 hours in patients with myocardial infarction and had returned to low levels by day 5. The prostaglandins produced by proliferating fibroblasts might be largely limited to the tissues and only slowly excreted. However, in the acute phase of infarction, the fibroblasts presumably would all be of the normal phenotype, and there would not have been time for the marked proliferation. Thus, we performed several experiments to determine whether fibroblasts might contribute to prostaglandin production in the very early phases of acute myocardial infarction. Work by Chien et al. has shown that arachidonic acid can be released from myocytes undergoing the metabolic changes seen in ischemia and infarction. In experiments with isolated canine cardiac myocytes, we confirmed these results by showing that previously incorporated [³H]arachidonate is released from the myocytes when they undergo calcium shock. We subsequently showed that the fibroblasts can take up the myocyte-derived arachidonic acid and incorporate it into their phospholipids. The distribution of this arachidonic acid is similar to that derived from exogenous free arachidonate. Subsequently, if the fibroblasts are stimulated, they are able to release the myocyte-derived arachidonic acid as efficiently as that derived from other sources. From these results, we propose that the fibroblasts that exist in normal myocardium may be stimulated to produce prostaglandins early in the course of acute myocardial infarction by the presence of large amounts of free arachidonate released from dying myocytes. If so, then fibroblast production of prostaglandins might contribute to the pathophysiological events seen very early in the course of myocardial infarction as well as those seen in the healing phase.

Acknowledgments

We thank Drs. Tom McIntyre, Guy Zimmerman, Ken Spitzer, and Kei Satoh for many helpful suggestions. We are grateful to Anthony R. Seeger and Kenneth Leichty for expert assistance in the performance of experiments and to Leona Archuleta and Linda Jara for help in preparing the manuscript.

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Key words: prostaglandins • myocardial infarction • fibroblasts • arachidonic acid
Arachidonate metabolism in cultured fibroblasts derived from normal and infarcted canine heart.
D R Weber, E D Stroud and S M Prescott

Circ Res. 1989;65:671-683
doi: 10.1161/01.RES.65.3.671

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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