Aldosterone and D-Glucose Stimulate the Proliferation of Human Cardiac Myofibroblasts In Vitro

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Abstract—The renin-angiotensin-aldosterone-system appears to be involved in the development of cardiac fibrosis in rodents, characterized by nonepithelial cell proliferation and changes in the extracellular matrix. The aim of our study was to investigate the effect of high aldosterone concentrations on the proliferation of human cardiac interstitial cells in vitro. In addition, the effect of D-glucose as another risk factor for fibrosis, e.g., in the diabetic heart, was investigated. Human cardiac myofibroblast cultures were established, and growth rates were measured by WST-1 assay in fetal calf serum–free Dulbecco’s modified Eagle’s medium (DMEM). Cells in culture showed a significant increase in number between 24 to 72 hours of cultivation under basal conditions (DMEM, 10% fetal calf serum). Aldosterone at high concentrations (10^{-8} and 10^{-7} mol/L) significantly (P<0.01) increased the proliferation of cultured cardiac myofibroblasts. Comparable effects were observed after incubation of the cells with high D-glucose concentrations (15 and 25 mmol/L, P<0.01). No additive growth stimulation was evident when the cells were incubated in medium containing both aldosterone and D-glucose. These results suggest a role for aldosterone and glucose in mediating the cardiac fibrosis through stimulation of myofibroblast growth in patients with dysregulated renin-angiotensin-aldosterone-system (especially hyperaldosteronism) and impaired glucose homeostasis. (Hypertension. 2002;39:756-760.)

Key Words: aldosterone ■ glucose ■ cardiac myofibroblasts ■ human

Recent clinical studies demonstrate a reduction in cardiovascular mortality in patients with heart failure treated with ACE inhibitors, angiotensin II blockers, and aldosterone antagonists. These effects were also seen in patients with diabetes mellitus. Ramipril reduced not only cardiac failure and myocardial infarction but also diabetic complications and diabetes mellitus. Ramipril reduced not only cardiac failure with ACE inhibitors, angiotensin II blockers, and aldosterone loop diuretics, digoxin, etc) had a beneficial effect on the cardiovascular system. Diffuse areas of fibrosis throughout the myocardium have been described, reflecting alterations in the microvascular system of the heart in diabetic patients, changes defined as diabetic cardiomyopathy. Post-

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mortem analyses of cardiac tissue from diabetic patients demonstrated interstitial and focal perivascular accumulation of collagen, indicating fibrosis. We therefore hypothesized that aldosterone and glucose may have synergistic stimulatory effects on the proliferation of myofibroblasts which could contribute to the excess cardiovascular mortality in diabetics.

Methods

Subjects
Patients (4 women, 3 men; age, 66.5±2.8 years; body mass index, 25.7±2.2 kg/m²; glycosylated hemoglobin, 5.7±0.2%) who underwent cardiac surgery for coronary artery bypass or valve replacement gave informed consent for biopsies from the right atrium. They were on comparable medications (diuretics, glycosides, ACE inhibitors, calcium-antagonists, β-blockers, nitrates). None of them had diabetes mellitus, although all had related conditions such as hypertension and dyslipidemia. The study was approved by the ethics committee of the medical faculty of the University of Leipzig.

Explanting Cardiac Biopsies
About 20 mg of human cardiac tissue was washed 5 times with 3 mL ice-cold PBS for 1 minute by gentle shaking. Fragments of 1 mm were placed on the bottom of dry cell culture flasks (75 cm² Greiner) at 37°C in humidified air with 5% CO₂ for 20 minutes. The tissue pieces were overlaid with 20 mL of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and incubated at 37°C in humidified air with 5% CO₂. The medium was changed every 72 hours. Subcultures were established from the cells grown in a 250-mL cell culture flask by overlaying 2 mL trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA in PBS). Trypsinization was stopped after 2 to 3 minutes by the addition of 10 mL of DMEM supplemented with 10% FCS. Cells from third and fourth passages were used for experiments.

Cell Proliferation Assay
For estimation of the proliferation rate, the cells were quantified by the nonradioactive colorimetric assay WST-1 (Boehringer Mannheim), based on the cleavage of a tetrazolium salt, as recommended by the manufacturer.

Experiments
Two subconfluent cell cultures on 24-well plates were incubated with medium containing different potential growth modulators. To ensure reproducibility, cells were incubated with 5, 15, and 25 mmol/L D-glucose; 5 mmol/L D-glucose plus 10 nmol/L aldosterone; 5 mmol/L D-glucose plus 100 nmol/L aldosterone; and 25 mmol/L D-glucose plus 100 nmol/L aldosterone and with 25 mmol/L L-glucose as control for osmotic and glycosylation effects for 24, 48, or 72 hours. The medium was changed every 24 hours, and proliferation was measured by WST-1 assay. Each experiment was repeated 8 times.

Cell Culture Characterization
The origin of the cells was identified by fluorescence-activated cell sorter (FACS) analysis using fibroblast-specific monoclonal antibodies mAb-1 and mAb-2. The monocytes and endothelial cells appearing in the culture were characterized by CD-14, CD-11, and CD-31 antibodies. Indirect immunofluorescence analysis with monoclonal antibody against smooth muscle α-actin (SMAA; Progen) was performed as described elsewhere.

Measurement of Glucose Utilization and Lactate Production
D-glucose and lactate concentrations of the medium were measured by glucose and lactate test kits (Sigma Diagnostics, Nos. 510-A and 826-UV) after incubation of the cells with 5 or 25 mmol/L D-glucose for 96 hours, and protein concentrations were measured according to the method of Bradford.

Statistical Analysis
All data are expressed as mean±SEM. Repeated measurement ANOVA was used to compare time-dependent data. Analysis of >2 groups of data were performed by 2-way ANOVA and, when required, by Bonferroni adjustment. Values of P<0.05 were considered statistically significant.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

FACS Analysis and Immunocytochemistry
The purity of the human cardiac myofibroblast cultures (2 to 3 passages) was confirmed by FACS analysis using the 2 fibroblast-specific antibodies, monoclonal antibody(mAb)-1 and mAb-2. FACS analyses of 2 cell lines from different patients were performed twice. The results showed high purity of the established cell cultures. Analysis of the worst case resulted in binding of 97.3% of the cells on fibroblast antibody mAb-1 and 99.7% on fibroblast antibody mAb-2 in comparison to low binding on the monocyte-specific antibodies CD-14 (0.5%) and CD-11 (0.4%), and the binding on the endothelial cell–specific antibody CD-31 (7.2%).

Under the light microscope the cells grown on the coverslips were heterogeneous in size and morphology. To establish the origin of these cells, mAb against SMAA, the marker of myofibroblastic differentiation, was used. Strong staining for SMAA was seen throughout the cytoplasm of the cells (Figure 1). The majority of the cells possessed filopodium- and lamellipodium-like structures projecting out of flat soma. Also, spindle-shaped cells and those with rodlike morphology could be observed.

Glucose Utilization and Lactate production
Glucose utilization and lactate production by human myofibroblasts (10 000 cells=0.6 μg protein) were measured after incubation with DMEM containing 5 or 25 mmol/L D-glucose on a 24-well plate containing 1 mL of the medium in each well. After 96 hours of incubation, a range of D-glucose utilization of 1.07±0.03 nmol/μg protein was observed. For
25 mmol/L δ-glucose, glucose utilization was nearly double (2.30±0.06 nmol/mg protein). This represents a reduction of initial δ-glucose concentration of <1% within 96 hours for both glucose concentrations. Lactate production after incubation of the cells in 5 mmol/L δ-glucose containing DMEM for 96 hours was 5.41±0.28 nmol/mg protein, whereas in the medium containing 25 mmol/L δ-glucose, lactate production was 6.99±0.21 nmol/mg.

Proliferation of Human Cardiac Myofibroblasts

To evaluate the proliferation of human cardiac myofibroblast cultures under basal conditions, cell numbers of 7 human cardiac fibroblast lines were measured after 24, 48, 72, and 96 hours by the WST-1 proliferation assay in DMEM containing 5% δ-glucose and 10% FCS. The mean of the WST-1 signals from all observed cell lines showed a significant increase in cell number between 24 and 48 hours (P<0.0001) and a further increase from 48 to 72 hours (P<0.0001). No change in cell number was seen between 72 and 96 hours (P>0.05).

The maximum increase in cell number was seen between 48 and 72 hours, with a doubling time of >70 hours.

Proliferation of Human Cardiac Myofibroblasts Stimulated by δ-Glucose

Supplementing FCS-free DMEM with additional glucose (15 and 25 mmol/L) increased human cardiac myofibroblast proliferation after 24 hours compared with those incubated in 5 mmol/L δ-glucose medium. This effect was absent when cells were incubated in FCS-free DMEM with L-glucose (25 mmol/L). The stimulatory effect of δ-glucose was seen after 24 hours (Figure 2) and at 48 and 72 hours (data not shown) of incubation time. There was no significant difference in proliferation of cells incubated with either 15 or 25 mmol/L δ-glucose (Figure 2).

Proliferation of Human Cardiac Myofibroblasts Stimulated by Aldosterone

Addition of high aldosterone concentrations (10⁻⁴ or 10⁻⁷ mol/L) to FCS-free medium for human cardiac myofibroblast cultures resulted in significantly higher numbers of cells compared with those incubated without aldosterone. The stimulatory effect of aldosterone was seen after 24 (Figure 3), 48, and 72 hours (data not shown) of incubation. There was no significant difference in proliferation between cells incubated with either 10⁻⁴ or 10⁻⁷ mol/L aldosterone (Figure 3).

Proliferation Stimulated by Coincubation of δ-Glucose and Aldosterone

Simultaneous incubation of human cardiac myofibroblasts with δ-glucose (25 mmol/L) and aldosterone (10⁻⁷ mol/L) in FCS-free DMEM resulted in a significant increase in cell number after 24 hours of incubation compared with those incubated in normal δ-glucose medium (5 mmol/L) without aldosterone. There was no synergistic effect of coincubation with 25 mmol/L δ-glucose plus 10⁻⁷ mol/L aldosterone compared with incubation with equimolar concentrations of aldosterone and δ-glucose alone (Figure 4).

Discussion

Cardiac remodeling leading to changes in the extracellular matrix, and in some cases to cardiac fibrosis, is a process involving myofibroblasts as an active component. We established primary cultures of myofibroblasts from human cardiac explants. The results of FACS analysis and the immunocytochemical investigations clearly demonstrate the myofibroblastic origin of the cells growing out of heart explants. Evidence for this was by (1) strong staining for SMAA, the main myofibroblastic marker protein of perivascular cells, and (2) morphological features, i.e., the well-defined leading-edge filopodium-
and lamellipodium-like structures, typical for activated myofibroblasts, the main producers of extracellular matrix. Although the outgrowth technique may lead to a selection of cardiac cells, the morphology of our myofibroblasts is very similar to fibroblasts obtained from human heart tissue treated by trypsin and collagenase digestion. Although in vivo studies strongly suggest direct aldosterone effects on fibroblasts, there is a surprising lack of information about the effect of aldosterone on cell proliferation. It has been reported that aldosterone increases the colony formation/proliferation of granulocyte-macrophage cells and diminishes the same parameters for erythroid cell proliferation. It has been reported that aldosterone contributes to cardiac hypertrophy and fibrosis in rats.9,24 Of intramyocardial sodium-dependent aldosterone synthesis, aldosterone action in vivo is supported by the recent finding and S. Neumann, 1994, unpublished observations). The myofibroblast cultures used in our experiments showed a proliferation stimulating activity of aldosterone at concentrations of 10^-8 mol/L aldosterone was without effect on incorporation of [3H]-proline into collagen,17 raising questions about the existence of mineralocorticoid receptors (MRs) in these cells. The presence of MRs and the 11β-hydroxysteroid-dehydrogenase in rat and human heart homogenates and myocytes was found to be a prerequisite for selective aldosterone effects.21 Evidence that MR and 11β-hydroxysteroid-dehydrogenase do exist in rat cardiac fibroblasts was provided by binding studies, which later were confirmed by immunohistochemical and biochemical studies; thus, molecular biological experiments demonstrate the expression of MR-mRNA.22,23 Myofibroblasts, preferentially found in areas of active collagen deposition, can synthesize collagen type I and III and contain SMAA.12 In addition, we observed that these cells have contractile features, characteristic of myofibroblasts when stimulated with angiotensin II (G.H. Scholz and S. Neumann, 1994, unpublished observations). The concept that these cardiac myofibroblasts may be the target of aldosterone action in vivo is supported by the recent finding of intramyocardial sodium-dependent aldosterone synthesis, contributing to cardiac hypertrophy and fibrosis in rats.24 Recently the expression of CYP11B2 (aldosterone synthase)-mRNA was detected in the failing but not in the normal human heart,25 supporting, on the molecular level, previous studies on activated intracardiac aldosterone production in humans with left ventricular systolic dysfunction.26

It has been reported that the myocardial aldosterone concentration in rats is about 1.6×10^-8 mol/L.9 This is 16-fold higher than the mean plasma aldosterone concentration in this animal. The myofibroblast cultures used in our experiments showed a proliferation stimulating activity of aldosterone at concentrations of 10^-7 and 10^-4 mol/L. That the lower aldosterone concentration has already maximum stimulatory effect on cell proliferation supports the involvement of classical MR effects. Intermediate early genes as of sgk (serum- and glucocorticoid-regulated kinase) are induced at 10^-8 mol/L aldosterone and can be blocked by ZK91587, a specific MR antagonist.27 This concentration also approaches that achieved in patients with primary and secondary hyperaldosteronism. Therefore, it seems reasonable to assume that there are at least 2 possibilities to explain the effect of aldosterone on the development of cardiac fibrosis in humans. First, such an effect may reflect enhanced synthesis of connective tissue components (collagen, fibronectin) by the individual fibroblast or myofibroblast; in addition, aldosterone may increase the number of myofibroblasts by promoting the proliferation of these cells.

In our experiments, high D-glucose concentrations stimulated the proliferation of myofibroblasts. This effect was not related to changes in osmolarity or to production of advanced glycosylation end products, because equimolar concentrations of nonmetabolizable L-glucose did not influence proliferation. A stimulatory effect of high glucose (10 to 30 mmol/L) was found in human dermal fibroblasts,28 arterial smooth muscle cells,29 and human renal cortical fibroblasts.29 Other groups reported an inhibitory or a toxic effect of high D-glucose on the proliferation of human dermal fibroblasts,30 which was prevented by aldose reductase inhibitors. A biphasic effect of enhanced D-glucose concentrations on the proliferation of mesangial cells was found, possibly related to a time-dependent change in the level and activity of paracrine factors.31 Recently published data have also shown a stimulating effect of high glucose concentrations on cellular proliferation of vascular smooth muscle cells, suggesting that transforming growth factor-β and protein kinase C (PKC) play an important role in this process.32 It seems possible that glucose, by activation of PKC, enhances the expression of c-fos and c-jun, which as components of the activator protein-1 (AP-1) complex are known to interact with the AP-1 binding site on the transforming growth factor-β receptor type II promoter.33 Apart from the potential role of paracrine factors, the experimental design may contribute to alternative results. A critical factor may be the time of incubation with glucose, which in confluent cultures can lead to substrate exhaustion and growth retardation. This is unlikely to be the case in our experiments; even in the presence of maximally growth stimulating 10% FCS, glucose utilization was low and the medium was changed every 24 hours. Because the experiments with high and normal D-glucose were done in FCS-free medium, even lower glucose utilization can be assumed. Taken together, the available data suggest that hyperglycemia might be able to induce fibrosis in the heart in different ways: via temporary or sustained stimulation of cells of the fibroblast lineage or via an increase in extracellular matrix production of these cells. To examine a possible synergistic effect of aldosterone and glucose on proliferation, we coincubated myofibroblasts with aldosterone and glucose. A stimulatory effect, and in some experiments even a synergistic effect, of aldosterone and glucose was observed.34 Later it was shown in rat neonatal cardiomyocytes that indeed high glucose is able to modulate aldosterone-induced protein synthesis and that one of the possible key regulators of glucose-mediated proliferation, PKC, is also a target of aldosterone action.35 In this study on human cardiac myofibroblasts in primary culture, we have demonstrated that high D-glucose concentrations, as well as subglucocorticoid but near-maximal aldosterone concentra-
tions, stimulate cell proliferation compared with that of the normal plasma concentration. This effect can also be seen in coculture experiments, although we cannot exclude the possibility that the supramaximal concentrations of both stimuli used in our experiments may have masked other additive, synergistic, or even antagonistic effects occurring at different stimulus thresholds. Especially in regard to the much lower aldosterone concentrations necessary for non-genomic aldosterone effects on IP3, intracellular calcium, and PKC occurring at 10^{-11} to 10^{-10} mol/L,36 which can also be activated by glucose.31 This kind of possible interaction has to be investigated in the future.

In summary cardiac fibrosis may be induced or aggravated by high n-glucose and aldosterone concentrations in vivo, which alone or in combination may stimulate myofibroblast proliferation and extracellular matrix production.

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In Vitro

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