Down-regulation of Akt/PKB in senescent cardiac fibroblasts impairs PDGF-induced cell proliferation

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Abstract

Objective: Cardiovascular diseases are the leading cause of death in the Western World, especially in the elder population. One pathophysiological component of cardiovascular disease is myocardial fibrosis, primarily derived from cardiac fibroblasts. Here we investigated the regulation of proliferation of fibroblasts from hearts of adult rats by platelet derived growth factor AA (PDGF-AA).

Methods: Cardiac fibroblasts were isolated from adult Wistar rats. PDGF-induced cell proliferation was analysed by FACS. PDGF-receptor numbers were analysed by receptor binding assays. Using differential display, differentially expressed kinases were identified during ageing in vitro and confirmed by Northern and Western blotting. Transient overexpression of IRES-GFP constructs was used to analyse the role of the akt kinase on proliferation by FACS. Results: During in vitro senescence/aging of primary fibroblasts, the growth response to PDGF-AA was greatly reduced without alterations in its receptor number or affinity and without changes in downstream signalling via the MAP-kinase pathway. By using a differential display strategy selective for protein kinases, we identified reduced expression of Akt-1 kinase (PKB-alpha) in senescent rat cardiac fibroblasts. These findings were supported by data showing reduced expression of Akt-1 in heart samples from old humans. Overexpression of activated Akt-1 almost completely reconstituted PDGF-AA dependent cell proliferation in aged fibroblasts. Conclusion: These results support an important role for Akt in senescence and regulation of cardiac fibroblast cell proliferation.

Keywords: Aging; Fibrosis; Signal transduction

1. Introduction

Within the elderly population, cardiovascular diseases are the leading cause of mortality in the Western world [1]. Aging in healthy humans is associated with a constellation of changes in the myocardium that reduce the reserve capacity of the heart to respond to a challenge [2]. Among them, fibrosis is a constant finding in senescent hearts in both rats and humans [3]. Since developing interstitial fibrosis (accumulation of extracellular matrix proteins like fibrillar collagen) essentially impairs the heart’s range of contractility, it is an important determinant of pathophysiology. As cardiac fibroblasts are responsible for the production of these extracellular matrix components, the growth regulation of this cell type is of major interest [4]. Cellular growth is commonly regulated by hormones and growth factors. In the case of fibroblasts, platelet derived growth factor (PDGF) is a major mitogen in serum [5] and its two chains, termed A and B, can combine to three different biologically active dimeric isoforms: AA, AB and BB. Studies with PDGF and PDGF receptor knockout mice have shown that PDGF is indeed an important...
Since cardiovascular disease correlates with age, we investigated the role of PDGF-AA during ageing using an in vitro model system. During passaging, fibroblasts in culture undergo a limited number of population doublings. After an initial period of growth, proliferative potential declines and viable, non-dividing, so-called replicative-senescent cells appear [8]. Evidence suggest that replicative senescence is related to organism ageing. Cells from short-lived species tend to senesce after fewer population doublings (PD) than cells from long-lived species, cells cultured from old donors tend to senesce after fewer PD than cells from young donors and cells from humans with hereditary premature ageing syndromes senesce more rapidly than cells from age-matched controls [9]. One major alteration during ageing of fibroblasts in vitro is their reduced proliferative capacity. Senescent cells are still able to divide upon serum stimulation, however polypeptide growth factors lose their mitogenic activity during ageing [10,11]. This may be due to the reduced expression of positive regulators of the cell cycle like cyclin-A/B and cdk-1/2, and increased expression of inhibitors like p21 [12-14]. But, in general, little is known about the regulation of signal transduction components during senescence.

We therefore decided to investigate early alteration in signal transduction pathways upon ageing in vitro. We identified protein kinase Akt to be differentially expressed during ageing and found a regulatory role of this kinase in proliferation of primary cardiac fibroblasts.

2. Methods

2.1. Material and chemicals

All chemicals were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany) unless otherwise indicated. The polyclonal anti-rat Akt and anti-phospho tyrosine antibody was purchased from Biomol (Hamburg, Germany), anti-phosphoglycosynthase-kinase-3 (GSK-3) was from New England Biololabs (Schwabach, Germany), Anti-ERK-2 from Life Technologies (Eggenstein, Germany) and the monoclonal anti-akt antibody from Sigma (Deisenhofen, Germany).

2.2. Myocardial infarction

 Coronary artery ligation was performed as described previously [15]. In brief, rats were anaesthetised by ether, intubated and ventilated by a volume-constant rodent ventilator (UB 7025 rodent ventilator, Hugo Sachs Elektronik, March, Germany), and a left thoracotomy was performed. The heart was exteriorised from the thorax, and the left coronary artery was ligated using a 5.0 suture between pulmonary artery outflow tract and left atrium. The heart was then returned to its normal position and the thorax closed. Animals were housed in polyethylene cages in climatized rooms with a 12-h light/dark cycle and fed with standard animal lab food and tap water. All procedures conformed to the guiding principles of the American Society of Physiology and were approved by the respective authorities.

2.3. Cell culture and cell transfection

Preparation and cell culture of primary cardiac ventricular fibroblasts from adult male Wistar rats were performed as described [16]. Briefly, two 300–400 g male Wistar rats were anaesthetised with 60 mg/kg ketamine hydrochloride (Ketavet, Parke-Davis, Berlin, Germany), 3 mg/kg xylacine (Rompun, Bayer AG, Leverkusen, Germany) and heparin (Thrombophob, Nordmark, Uetersen, Germany). The hearts were excised, mounted on a Langendorff system and rinsed with perfusion buffer (PB), containing in mM: NaCl 110, KCl 2.6, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25, glucose 11 at 37°C continuously gassed with 95% O2/5% CO2. Perfusion was continued by recirculating PB for 20 min, containing 0.25 mg/ml collagenase P (Boehringer, Mannheim, Germany) and 12.5 μM CaCl2. The ventricles of the hearts were then cut with a tissue chopper in 0.7×0.7 mm pieces and incubated for another 10 min in 30 ml reperfusion buffer, containing 400 mg BSA. The material was filtered through a 250-μm nylon mesh, spun down at 25×g for 3 min and the resulting pellet was resuspended and centrifuged another two times at 25×g for 3 min. Afterwards, the supernatants of the three centrifugation steps were collected and spun down at 250×g for 5 min. The pellet was resuspended in McCoy-5A medium/10% calf serum and plated on six 12-well plates (Falcon, BD, Heidelberg, Germany). Two to four hours later, the debris and nonattached cells were removed by medium changes. The medium was changed the next day and the cells were grown for 5 days in McCoy-5A medium containing 10% Hyclone calf serum without another medium change. The culture contain more than 90% fibroblasts. Fibroblasts were passaged 1:4 once a week.

The BglII/BamHI fragments of myr-akt, WT-akt, akt(K179M) and akt(PH) (special gift of T. Franke, New York, USA) were cloned into the pEGFP-RES-2 vector from Clontech. Cardiac fibroblasts were transfected with 1 μg plasmid DNA using Fugene (Boehringer Mannheim, Germany) according to the manufacturer’s protocol, or electroporation using hypoosmolar buffer (100 μs, 4000 V/cm). One day after transfection, medium was changed to MCDB-402 without serum for 24 h (synchronisation).
After an additional medium change, cells were stimulated for 24–48 h with PDGF. Transfection efficiencies were about 5%.

2.4. Human heart samples

Samples from the left ventricles were taken during a post mortem examination usually performed 24–36 h after the legal declaration of death. In the meantime corpses were stored at 4°C. The removed tissue samples were immediately frozen in liquid nitrogen. All procedures were performed according to the guidelines outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–3).

2.5. Cell cycle analysis

Cells were fixed for 10 min with 2% paraformaldehyde at room temperature, treated with 0.1% Triton X-100, stained with 1.2 µg/ml Hoechst 33258 and incubated for 15 min at 4°C in the dark. Cell cycle analysis was done, using a 325-nm helium–cadmium laser for the activation of the Hoechst dye (cell cycle analysis) and a 488-nm argon laser (gfp expression as a measure for Akt) to distinguish between Akt expressing and non-expressing cells. Cells were analysed on an Coulter Epics Elite flow cytometer (Coulter, Krefeld, Germany). All data were Listmode recorded and quantified using the software Multi2D and MultiCycle (Phoenix Flow Systems, San Diego, USA).

Analysis of variance was used to determine significant differences of the fraction of proliferating cells between the groups. Subsequently, a Bonferroni t-test was used to compare the different forms of Akt expressing cells with the control cell population. P<0.05 were considered significant.

2.6. RNA preparation and Northern blotting

Total RNA was isolated according to Chomczynski and Sacchi [17] and Northern blotting was done as described [18]. Briefly, 10 µg total RNA were denatured at 65°C in an solution containing 1.2 M formaldehyde, 35% formamide, and ethidium bromide. The RNA was electrophoresed in a 1.25% agarose gel containing 6.7% formaldehyde. After transfer by vacuum onto nylon sheets (Hybond N, Amersham, Freiburg, Germany) in 6× SSC (1× SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0) the RNA was crosslinked by UV irradiation (1 mJ/cm² for 30 s). The blot was hybridised at 60°C in 5× SSC, 10% Denhardt’s solution, 0.1% SDS, 300 µg/ml denatured herring sperm DNA: The DNA probes were labelled using an oligolabeling kit from Pharmacia (Freiburg, Germany) with [α-32P]dCTP (Hartmann Analytic, Braunschweig, Germany) The filters were washed in solutions containing a decreasing fraction of SSC in 0.1% SDS and 2 mM EDTA. The final wash solution contained 0.4% SSC. Radioactivity was detected and quantified by a phosphoimager. The probes used were the family specific PCR products reamplified from the differential display reaction.

2.7. Western blotting

Cell or tissue samples were lysed in buffer containing 50 mM Tris pH 7.5, 2% SDS, 1 mM Na₂VO₄, 10 µM Leupeptin, 1 mM PMSF and 100 mM okadaic acid. After protein content estimation, 0.05% mercaptoethanol was added, the probes heated to 70°C for 5 min and frozen. Western blotting was performed as previously described by Simm et al. [16]. Briefly, after SDS–PAGE (20 µg protein/ lane) proteins were transferred onto reinforced nitrocellulose by semidy blotting. The sheets were saturated with BSA and incubated for 1 h with rabbit polyclonal anti-rat Akt kinase (1 µg/µl, Biomol, Germany) antibodies. After washing, goat anti-rabbit IgG horseradish peroxidase-labelled antibody (1:10 000, Dianova) was added for 1 h. The detection was done by chemiluminescence, using luminol (2.5 mM) and P-coumaric acid (400 µM) as enhancer. The blots were reprobed using monoclonal anti rat actin antibodies (Sigma) as loading controls.

2.8. Family-specific domain-primed differential display (FSD-DD)

Total RNA was isolated according to the method of Chomczynski and Sacchi [17] and 5 µg of total RNA were reverse transcribed in a 15 µl reaction mixture containing (in mM): 50 Tris–HCl (pH 8.3), 50 KCl, 4 MgCl₂, 10 DTT (MBI Fermentas, Heidelberg, Germany), 0.33 of each dNTP (Appligene, Heidelberg, Germany), 20 Units of RNAsin® (Promega, Heidelberg, Germany), 15 pmol of an oligo-dT₁₇ primer, and 10 Units of MMLV-reverse transcriptase (MBI Fermentas, Heidelberg, Germany). After a 60-min incubation at 37°C, 2 µl served as template in the first PCR which introduces the family-specific domain primer (FSD-primer, see Table 1). The 50-µl PCR mixture included (in mM): 20 Tris–HCl (pH 8.5), 16 (NH₄)₂SO₄ and 2.5 MgCl₂, 1.25 Units Taq polymerase (AGS, Heidelberg, Germany), 0.02 of each dNTP, and 30 pmol FSD primer. Then, 25 cycles were performed (30 s at 95°C, 1

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<th>Table 1</th>
<th>Sequences of primers used in the FSD-DD</th>
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<tr>
<td>Specificity</td>
<td>Sequence</td>
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<tr>
<td>FSD primer 1</td>
<td>CNG TNG AYT GGT TGG</td>
</tr>
<tr>
<td>FSD primer 2</td>
<td>CHG TGG AYT GGT GGG C</td>
</tr>
<tr>
<td>HpaII linker</td>
<td>CGG TGG CTT TCG GGC C</td>
</tr>
<tr>
<td>Anti-linker primer</td>
<td>GAT TCT CAA CCC GAA AGC CA</td>
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* N=(A, C, G, T); Y=(C, T); H=(A, C, T).
min at 56°C, and 2 min at 72°C). The second PCR mixture (50 μl) contained (in mM): 20 Tris–HCl (pH 8.5), 16 (NH₄)₂SO₄ and 2.5 MgCl₂, 0.05 of each dNTP, 15 pmol Taq primer as well as 15 pmol FSD primer and 1 Unit Taq polymerase, and 30 amplifications were performed (30 s at 95°C, 1 min at 40°C, and 2 min at 72°C). After purification, DNA was cleaved with 10 Units HpaII (MBI Fermentas, St. Leon-Rot, Germany) for 3 h at 37°C followed by heat-inactivation at 65°C for 30 min. Then, the purified restriction fragments were ligated at 16°C for 12 h to 66 pmol linker using 6 Units T4 DNA ligase (New England Biolabs, Schwalbach, Germany) in 15 μl buffer (50 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, and 2.5 ng BSA). After purification, the fragments framed by the FSD primer and linker were amplified in a third PCR using 30 pmol of both anti-linker primer and FSD primer (25 cycles, 30 s at 95°C, 1 min at 56°C, and 1 min at 72°C). The last PCR contained 3 pmol [γ-³²P]ATP (Hartmann Analytic, Braunschweig, Germany) labelled anti-linker primer, 0.4 Units Taq polymerase and 10 μl DNA template from the third PCR. Amplification consisted of 30 cycles (30 s at 95°C, 1 min at 56°C, and 1 min at 72°C). DNA was loaded onto a 6-M denaturing urea-sequencing gel to separate the amplified fragments. After exposure for 8 days, bands of interest were cut out and reamplified. PCR products were cloned into the pBluescript KS+ vector (MBI, Fermentas). All PCR products were sequenced using the fluorescent di-deoxy terminator method of cycle sequencing on a Perkin Elmer, Mass spectrometry. The sequences were analysed by aligning them to the published sequences of PDGF receptor α and β subunits using CLUSTALW and GeneDoc software.

2.9. PDGF receptor binding assays

PDGF receptor binding assays were carried out as described [19]. Briefly, cardiac fibroblasts were grown in 12-well plates and synchronised with MCDB-402 medium as described above under cell culture. The cells were placed on ice and washed two times with binding buffer (MCDB, 20 mM HEPES, 1 mg/ml BSA, pH 7.2). Increasing amounts (1–50 ng/ml) of [³²P]PDGF isoforms were added to the wells. For each concentration of [³²P]PDGF, the amount of unspecific binding was estimated separately by the addition of 1 μg/ml unlabelled ligand. The plates were incubated at 4°C for 4 h, washed five times with washing buffer (PBS with Ca²⁺ and Mg²⁺, 1 mg/ml BSA), and lysed in 1 ml of lysis buffer (1% Triton X-100, 10% glycerol in 20 mM Tris–HCl, pH 7.5). The lysate was counted in a gamma-counter.

2.10. Akt-kinase assay

The kinase assay was performed using a commercial kit according to the manufacturers instructions (New England Biolabs, Schwalbach, Germany). Briefly, quiescent fibroblasts were stimulated with 50 ng/ml PDGF-AA for 10 min and subsequently lysed in buffer containing 20 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₄, 1 μg/ml leupeptin and 1 mM PMSF. After centrifugation, 250 μl lysate containing 250 μg protein was incubated with immobilised Akt-antibody slurry overnight at 4°C, microcentrifuged and washed twice with lysis buffer. The pellet was washed twice with kinase buffer (25 mM Tris pH 7.5, 5 mM glycerophosphate, 2 mM DTT, 0.1 mM Na₂VO₄, 10 mM MgCl₂) and the reaction started with kinase buffer containing 1 μg GSK-3α fusion protein and 200 μM ATP. After 30 min at 30°C, the reaction was terminated by 3× SDS buffer and phosphorylation of the substrate was analysed by Western-blotting, using an anti-phospho GSK-3α antibody.

3. Results

As shown in Fig. 1, both PDGF-AA and PDGF-BB stimulate cell proliferation in primary non-passaged cardiac fibroblasts. After one passage in vitro, the mitogenic capacity of both PDGF isoforms was lowered by about 30%. Furthermore, we found a remarkable shift in the EC₅₀ value for PDGF-AA. Whereas non-passaged cardiac fibroblasts required 8.5 ng/ml PDGF-AA for a half-maximal proliferative response, passaged cells required 24.5 ng/ml. After four to six passages, both PDGF isoforms failed to stimulate cardiac fibroblast growth at all (data not shown). These in vitro data appears to have in vivo relevance since similar results were obtained using primary non-passaged cardiac fibroblasts from heart-infarcted rats (Fig. 1, lower panel), i.e. the EC₅₀ values for PDGF-AA were higher than those obtained from non-infarcted rats. A possible explanation for this shift in the EC₅₀ value could be altered receptor numbers or binding affinities.

Binding assays were performed to determine the number of receptors and the receptor binding affinity for both PDGF isoforms before and after in vitro passaging of cardiac fibroblasts from control rats (Fig. 2). PDGF-AA binds exclusively PDGF α-receptors whereas PDGF-BB binds mainly β-receptors. Neither the number of PDGF-AA receptors (15 300±1800 vs. 15 000±70) nor the binding affinity (177±72 vs. 159±1 pM) changed significantly after passaging. The PDGF-BB binding assay revealed similar results. Both receptor number (24 800±1800 vs. 29 500±2500) and binding affinity (387±136 vs. 397±37 pM) do not show significant alterations after passaging. The binding affinities for the α- and the β-receptor are consistent with data reported by Hoppe et al. [19] in a fibroblast cell line and Booz and Dostal [20] in neonatal cardiac fibroblasts.

To confirm activation of the PDGF α-receptor, non-passaged and passaged fibroblasts were stimulated with PDGF-AA and receptor autophosphorylation was analysed
Fig. 1. Alterations in PDGF induced cell proliferation during ageing in vitro and in vivo. Cardiac fibroblasts were grown to confluence, synchronised with medium without serum for 2 days and stimulated with the indicated amounts of PDGF for 48 h. Cell proliferation was analysed using the BrdU/Hoechst quench method. In panel A, primary fibroblasts were used. In panel B, cells were used after one or two passages in vitro. Panel C, primary non passed cardiac fibroblasts from rats after infarction were used. Whereas non-passaged and non-infarcted cardiac fibroblasts needed 8.5 ng/ml for half-maximum proliferative response, passaged cells required 24.5 ng/ml PDGF-AA. Non-passaged cells from infarcted hearts also show this shift in the EC_{50}-value.

by Western blotting. Using anti-phospho tyrosine antibodies, we have shown earlier that 10 min PDGF-AA causes a maximal activation of α-receptors observed as autophosphorylation of a 180-kDa receptor band [21]. As shown in Fig. 3A, there are no major differences seen in the tyrosine phosphorylation of the α-receptors, represented by the 180-kDa band. In addition, in both the non-passaged and passaged cells PDGF-AA stimulated activation of ERK kinases with the same kinetics as shown by the retarded mobility of the phosphorylated active p42-MAP kinase (ERK2) (Fig. 3B). We could also show that ERK was activated by PDGF-AA within the same concentration range in non-passaged and passaged cells (Fig. 3C). This activation indeed does not correlate with the results of the cell proliferation assay in Fig. 1.

One way to address the reduced growth capacity of the senescent cells is to screen for differentially expressed genes. We used the FSD-DD technique (family-specific domain-primed differential display) [22], for studying the differential expression of serine/threonine kinases, a major family of kinases within the signal transduction cascades. Due to a restriction and ligation step, this method usually yields short (100–200 bp) DNA fragments within the coding region. Using this method, we identified three differentially expressed kinases, Akt-1 (also termed PKB-alpha and RAC), nucleoside diphosphate kinase (NDPK), and pyruvate kinase. Furthermore, as a control, we amplified a band that was not differentially displayed and found another serine/threonine kinase termed protein kinase N (PKN). As shown in Fig. 4, the differential expression of two kinases, Akt-1 and pyruvate kinase, could be verified by Northern blotting.

As Akt is involved in the regulation of several signalling pathways, we decided to investigate the role of this kinase in more detail. Akt-1 is activated upon ligation of several cell surface receptors such as the PDGF receptor. Akt’s biological significance has been demonstrated by its ability to protect various cell types from apoptosis such as neurons. On the other hand, little is known about the role of Akt on cell proliferation [23].

To confirm the differential expression at the protein level, we performed Western blotting (Fig. 4B). We verified the differential expression of Akt-1/PKB-alpha by a factor of two, which resembles the situation on the level of the RNA (Fig. 4A). In addition, we were not able to detect a difference in the expression of Akt-2/PKB-beta at the protein level (data not shown). To ensure not only a differential expression but also a differential activation of
The EC\textsubscript{50} value (25 ng/ml) for PDGF-AA induced proliferation of passaged control cells was used. At this concentration, PDGF-AA induced proliferation by 18% in passaged and about 50% in non-passaged cells. Over-expression of wild-type Akt (wt-Akt) or myristoylated-Akt (myr-Akt) increased the proliferative response of passaged cells to about 30 or 40%, respectively (Fig. 7). Using FACS analysis, we determined that PKB was overexpressed about 4-fold in the transfected passaged cells (data not shown). This expression level is twofold higher that that of PKB in non-passaged primary cells.

4. Discussion

We could show here, that reduced Akt expression is a component of senescence in rat fibroblasts and possibly also in human heart. Commonly, ageing is studied in vitro using fibroblasts as a model system. Most higher eukaryotic cells that can divide in vivo cannot do so indefinitely due to a process called cellular or replicative senescence [8]. Replicative senescence was first described for human fibroblasts in culture [24]. Cells that have reached the end of their replicative life span remain viable and metabolic active for long periods of time [25]. A lot of genes involved in cell cycle progression like cdk-1 / 2, cyclin-A / B, c-fos, and pol\textsubscript{a} are repressed during senescence [12,13,26,27]. This may contribute to the discussed function of the replicative senescence as a powerful tumour suppressive mechanism [9]. On the other hand, genes involved in the production and remodelling of the extracellular matrix as for example collagen \alpha\textsubscript{1}, fibronectin, collagenase and stromolysin are overexpressed by senescent (passaged) human fibroblasts and may thus contribute to fibrosis [28,29].

Little is known about early changes during the process of cellular ageing. In our model, we could not detect significant changes at the PDGF receptor level. This is in line with the results of Winkles et al. [30] who detected PDGF receptors and PDGF induced receptor autophosphorylation in premature aging progeria in comparison to normal fibroblasts. We here detect the Akt-1 / PKB-alpha as a differentially expressed signal transduction kinase. Akt-1 is a serine/threonine kinase downstream of the PI-3 kinase. It is phosphorylated and thereby activated by PDK1 which in term is activated by PtIns-3,4-bisphosphate, a product of the PI-3 kinase [31]. Many research groups refer to glycogen synthase kinase-3 as a major target of Akt [32]. The most prominent role of Akt is its function as a survival factor [33,34]. In addition, Akt activates the p70\textsuperscript{56} kinase, which was shown to be pre-activated in quiescent primary cardiac fibroblasts [16,35]. We report here that Akt-1 expression is reduced during passaging of primary cardiac fibroblasts in vitro. This finding from an in vitro rat cell culture are supported by data obtained with heart samples from old and young Akt, cardiac fibroblasts were stimulated with PDGF-AA, the Akt kinase immunoprecipitated and the kinase activity analysed using glycogen-synthase-kinase as a substrate. As shown in Fig. 5, Akt-1 was 3-fold stimulated in non-passaged cells whereas only a 1.75-fold increase could be detected using passaged cells.

In order to obtain more evidence for a possible pathophysiological role of the reduced Akt expression, Akt-1 expression was determined in human cardiac tissue taken post mortem. The samples were taken from hearts of patients without cardiovascular diseases. After staining for Akt-1 and actin, the ratio of both was used to calculate an expression level. The data shown here indicate that the expression-level of Akt is reduced by a factor of 2 in old versus young hearts (Fig. 6). This is in the same range that we found in vitro.

We then overexpressed Akt-1 in passaged cardiac fibroblasts and tested PDGF-AA induced cell proliferation.
Fig. 4. Akt-1 is differentially expressed at the mRNA and protein level. Panel A shows the differential expression of Akt-1 and pyruvate kinase. Nucleoside diphosphate kinase and protein kinase N were expressed at the same level in non-passaged (N) and passaged cells (P). Panel B shows the differential expression of Akt at the protein level between non-passaged and passaged cells from three independent experiments.

patients. The low sample number of samples is due to the fact that in this study only heart samples from patients without cardiovascular diseases were included. It can be postulated that Akt has a modular function. It was shown that Akt/PKB is upregulated during terminal differentiation. For example, Akt expression is low in a multipotent fibroblast cell line (10T1/2), but is increased when these cells are transformed with MyoD to induce differentiation into myocytes [36]. 3T3-L1 fibroblasts spontaneously differentiate into adipocytes when transfected with a constitutively active Akt [37]. On the other hand, we show here that Akt is down-regulated during ageing or cellular senescence. In line with our results, it was shown that an inhibitor of the upstream PI-3 kinase of PKB was able to induce cellular senescence [38].

Does Akt regulate cell proliferation? Most of the published data until now dealt with the function of Akt as an anti-apoptotic agent. Akt was detected as a cellular homologue of the viral oncogene of the retrovirus AKT8. It was discussed as playing a role in formation of a variety of tumours [39]. Due to its ability to regulate the p70S6 kinase it may modulate and influence protein translation (Fig. 8) [35]. Akt can also directly phosphorylate CREB and thereby modulate its transcriptional activity [40]. Glycogen synthase kinase 3 (GSK-3) another known substrate of Akt, phosphorylates transcription factors such as AP-1 [41]. Phosphorylation of GSK-3 by Akt leads to its inactivation and thereby blocks in the case of AP-1 the inhibitory effect of GSK-3. In addition, GSK-3 specifically phosphorylates cyclin D1 on Thr-286, thereby triggering rapid cyclin D1 turnover. It can also induce redistribution of cyclin D1 from the cell nucleus to the cytoplasm [42]. Therefore, cyclin D1 cannot bind to the appropriate cdk which leads to cell cycle block. Due to this inhibitory effect of GSK-3 on transcription factors and cyclin D1 stability, inactivation of GSK-3 by Akt should be able to increase cell proliferation. On the other hand, inhibition of PI-3 kinase, the most important upstream regulator protein of Akt, by wortmannin usually does not inhibit cell proliferation within cell lines [18,43]. In contrast to these findings using primary cells, it was shown that inhibition of the p70S6 kinase as a downstream target blocks cell division [16]. Thus, the cell type used for these studies has great significance. Primary cells have on the other hand disadvantages. The transfection efficiencies are very low and biochemical studies are difficult or impossible to do. Thus we are planning to use adenoviral constructs to further investigate the function of Akt/PKB in more detail in vitro.

Recent publications using Caenorhabditis elegans as an in vivo model clearly indicate involvement of Akt in the regulation of growth and senescence. The C. elegans homologs of mammalian PI-3-kinase (AGE-1), PDK-1 (pdk-1) and Akt (akt) regulate C. elegans longevity and
Fig. 5. Akt-1 is differentially activated after PDGF stimulation. Panel A shows an Akt kinase assay from PDGF-AA treated cardiac fibroblasts as described in Methods. The phosphorylation of the substrate glycogen-synthase-kinase 3 by Akt is clearly reduced after passaging (P) in comparison to non-passaged (N) cells. Panel B displays the results of three independent Akt-kinase assays. Shown are the means±standard deviations.

Fig. 6. Expression of Akt in human hearts. Left ventricular samples were taken post mortem from patients free of cardiovascular diseases. The upper panel represents the different Akt-1 expression between young (up to 70 years) and old (over 80 years) people as confirmed by Western blotting. The actin expression remained constant and the middle panel shows the calculated expression after measuring the density of the bands. The lower panel reflects the statistical significance using unpaired Student’s t-test. Two-tailed P<0.05 were considered significant.

eexpression of Akt is also responsible for differences during ageing in the atria and the ventricles. For example, prominent collagenous septa develop in human atrial pectinate bundles with ageing, and these septa appear to have a major effect on conduction of the electrical impulse. This has not been reported to our knowledge with ageing of the otherwise normal ventricle, even though the total ventricular collagen content increases [49]. In addition, atrial fibrillation is the most common arrhythmia in the geriatric group. Nakajima et al. [50] recently reported transgenic mice studies that demonstrated that although similar levels of active TGF-β1 were present in the transgenic atria and ventricles, overt fibrosis was observed only in the atria. One may speculate that various stress in
the atria and ventricles during life will cause an unequal amount of cell proliferation in vivo and therefore fibroblasts in a different state of cellular ageing. This would indeed cause, as we have shown here, unequal cellular responses upon similar growth factor stimulation.

In summary, our data indicate an important role for Akt during cellular senescence in vitro. This is supported by data obtained from human heart samples. Clearly, Akt is not the only factor responsible for ageing. The precise causal role of this kinase in regulating senescence and fibrosis will have to be determined in future experiments, which include use of Akt-deficient and Akt-overexpressing animals.

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Fig. 7. Overexpression of constitutively active Akt and wild-type Akt mainly reconstitute the PDGF-AA induced cell proliferation. Overexpression of constitutively active Akt-1 (myr-Akt) and wildtype Akt-1 (HA-Akt) results in a significant increase in cell proliferation ( ** P<0.01, * P<0.05). Overexpression of the PH domain of Akt or inactive Akt kinase (HA-Akt (K179M)) had no effect on PDGF-AA induced cell proliferation.

Fig. 8. Model for the role of Akt/PKB in cellular senescence. Downregulation of Akt can contribute to the reduced proliferative capacity during cellular senescence due to its ability to affect translation, transcription and the activity of cell cycle controlling kinases (cdks) (→: activation; ↓: inhibition).


