Apoptosis is triggered by the cyclic AMP signalling pathway in renal mesangial cells

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Abstract Glomerular mesangial cells are regarded as specialized smooth muscle cells located within the renal glomeruli and fulfilling important functions in glomerular physiology and pathophysiology. Here, we report that activation of the cyclic AMP signalling pathway by dibutyryl cyclic AMP, forskolin, or the β_2-adrenergic receptor agonist salbutamol results in induction of apoptosis in mesangial cells. Activation of the apoptotic programme results in DNA fragmentation which is visible for most forms of apoptosis and is paralleled by enrichment of cytosolic DNA/histone complexes, an increasing number of cellular 3′-OH-fragmented DNA ends and typical nuclear chromatin condensation. Induction of apoptosis was found to be dependent on translation and independent of nitric oxide synthase activity.

Key words: Apoptosis; Cyclic AMP; Forskolin; Salbutamol; Mesangial cells

1. Introduction

Apoptosis is a controlled biological strategy to remove unwanted cells from a given tissue and thus is involved in physiological and pathophysiological processes, e.g. limb formation during development, removal of self-reactant or hyperactivated immune cells as well as of virus-infected and tumour cells. Apoptosis is characterized by an ordered sequence of cellular events which include cellular shrinking, nuclear condensation, and disintegration of the dying cell into apoptotic bodies which are rapidly taken up by neighbouring cells. Thus the leakage of cell constituents with secondary inflammation is prevented. In most cellular systems apoptosis is associated with the activation of endonucleases which cleave double stranded DNA at the internucleosomal linker region thereby producing mono- and polynucleosomal DNA fragments. These fragments give rise to the typical DNA ladder which consists of multiples of about 180 bp units, regarded a biochemical marker of apoptotic cell death [1,2].

Mesangial cells located within the renal glomerulus are regarded as specialized smooth muscle cells, involved in the regulation of the glomerular filtration rate [3]. Moreover, once activated in a proinflammatory context mesangial cells release a variety of proinflammatory mediators and growth factors, thus being an important determinant of the immunological status of the renal glomerulus and contributing to glomerular pathophysiology [4]. Since apoptosis of mesangial cells was detected during the course of experimentally induced glomerulonephritis [5] this study was undertaken to further characterize cellular signals which initiate the apoptotic programme in mesangial cells. Here we present evidence that activation of the cyclic AMP signalling pathway results in mesangial cell apoptosis in a translation-dependent and nitric oxide synthase-independent manner.

2. Materials and methods

2.1. Chemicals

Salbutamol was generously supplied by Dr. Irmgard Wiesenberg, Ciba-Geigy Ltd., Basel, Switzerland; forskolin was from Calbiochem (Lucerne, Switzerland); N^G-monomethyl-L-arginine (L-NMMA) was from Alexis (Läufelfingen, Switzerland). Cell death detection ELISA and in situ cell death detection kit were from Boehringer Mannheim (Rotkreuz, Switzerland). Cell culture media and nutrients were obtained from Gibco BRL (Basel, Switzerland) and all other chemicals were either from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) or Sigma (Buchs, Switzerland).

2.2. Cell culture

Rat mesangial cells were cultured, cloned and characterized as described previously [6]. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and bovine insulin (0.66 units/ml). For the experiments passages 10–18 of mesangial cells were used.

2.3. Analysis of DNA fragmentation by DNA agarose gel electrophoresis

Confluent mesangial cells in 85 mm diameter dishes were washed twice with PBS and incubated in DMEM without phenol red containing 0.1 mg of the fatty acid free BSA/ml with the indicated concentrations of compounds. Thereafter cells were lysed in lysis buffer (50 mM Tris/HCl pH 7.5, 20 mM EDTA, 1% NP-40) and apoptotic DNA fragments were isolated [7]. Electrophoresis was performed in a 1.5% agarose gel and DNA was stained using ethidium bromide (1 μg/ml). Photographs were taken by UV transillumination.

2.4. Cell death detection by analysis of cytosolic oligonucleosome-bound DNA

Confluent mesangial cells in 35 mm diameter dishes were washed twice with PBS and incubated in DMEM. Cytosolic oligonucleosome-bound DNA was quantitated using an ELISA kit with a primary anti-histone antibody and a secondary anti-DNA antibody coupled to peroxidase according to the manufacturer's instruction. Absorbance values ($A_{405nm/486nm}$) are a relative measure for DNA fragmentation. Percentage of fragmentation is expressed in comparison to controls [8].

2.5. In situ detection of apoptosis

Cells grown on glass plates were treated with different agents, washed with PBS, and fixed with 4% formaldehyde-containing PBS. Mesangial cells were blocked for 15 min with 0.3% H_2O_2 in methanol, washed and permeabilized for 2 min on ice with 0.1% sodium citrate in PBS. Apoptotic cells were detected in situ by enzymatically labelling the free 3′-OH DNA terminus using an in situ cell death detection kit. The reaction mixture was added for 60 min at 37°C. Following further washing the glass plates were incubated for 30 min at 37°C with anti-fluorescine antibody coupled to peroxidase. As chromomere

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0.05% diaminobenzidine in 1% nickel sulfate and 0.01% H_2O_2 was added for 15 min. Stained cells were dehydrated with increasing amounts of alcohol, dipped in xylol, and coverslips were sealed with Eukitt and evaluated by interference microscopy [9].

2.6. Staining of nuclei with Hoe-33258

Confluent mesangial cells in 35 mm diameter dishes were washed twice with PBS and incubated in DMEM with the indicated concentrations of compounds. Thereafter the cells were fixed in 3% paraformaldehyde in phosphate buffer (100 mM, pH 7.4). From a stock solution Hoe-33258 (1 mg/ml) was added up to a final concentration of 30 μg/ml. The nuclei were viewed under a fluorescence microscope using standard excitation filters [10].

3. Results

Analysis of DNA by gel electrophoresis reveals internucleosomal DNA fragmentation in mesangial cells exposed to either dibutyryl cyclic AMP (Fig. 1a), forskolin (Fig. 1b) or salbutamol (Fig. 1c), which all increase intracellular cyclic AMP levels either directly, by immediate activation of the catalytic subunit of the adenylyl cyclase, or by a β_2-adrenergic receptor coupled mechanism. Characteristic apoptotic DNA fragmentation was almost undetectable in unstimulated control cells. The forskolin analogue 1,9-dideoxyforskolin, which is inactive as a stimulator of adenylyl cyclase, has no effect on DNA fragmentation. Induction of DNA laddering was found to be dependent on protein synthesis since incubation of the cells with forskolin in combination with cycloheximide efficiently blocked DNA fragmentation (Fig. 1c). We have recently shown that cyclic AMP induces the expression of nitric oxide synthase in mesangial cells [11,12]. Furthermore, we have observed induction of apoptosis in mesangial cells exposed to exogenous nitric oxide donors (H. Mühl, K. Sandau, B. Brüne, J. Pfeilschifter, unpublished observations). Therefore we investigated next whether activation of apoptotic DNA degradation by the cyclic AMP signalling pathway is dependent on induction of nitric oxide synthase activity in mesangial cells. Cells exposed to forskolin release small but significant amounts of nitric oxide [11]. However, blockage of nitric oxide synthase activity by L-NMMA did not inhibit forskolin induced DNA fragmentation indicating that nitric oxide is not required for cyclic AMP-induced apoptosis (Fig. 2). DNA fragmentation was paralleled by enrichment of cytosolic DNA/histone complexes in response to elevated levels of cyclic AMP (Table 1). Again cycloheximide efficiently antagonized apoptosis indicating translational dependence (Table 1). To further extend these results to the level of the single cell we performed in situ detection of apoptosis by labelling the cellular free 3′-OH DNA ends using terminal deoxynucleotidyl transferase (TUNEL staining). Exposure of mesangial cells to forskolin results in a massive increase of TUNEL positive cells which further confirms cyclic AMP- dependent apoptotic DNA degradation (Fig. 3). Chromatin condensation which may occur independent of DNA fragmentation [13] is another characteristic feature of ongoing apoptosis. Nuclear staining of mesangial cells treated with forskolin revealed typical apoptotic chromatin condensation (Fig. 4) which often co-localized with budding at the cell membrane, visible by light microscopy. After a 24 h incubation with forskolin (20 μM) approximately 10% of the cells exhibited condensed chromatin which is not detectable in untreated mesangial cells (Fig. 4).

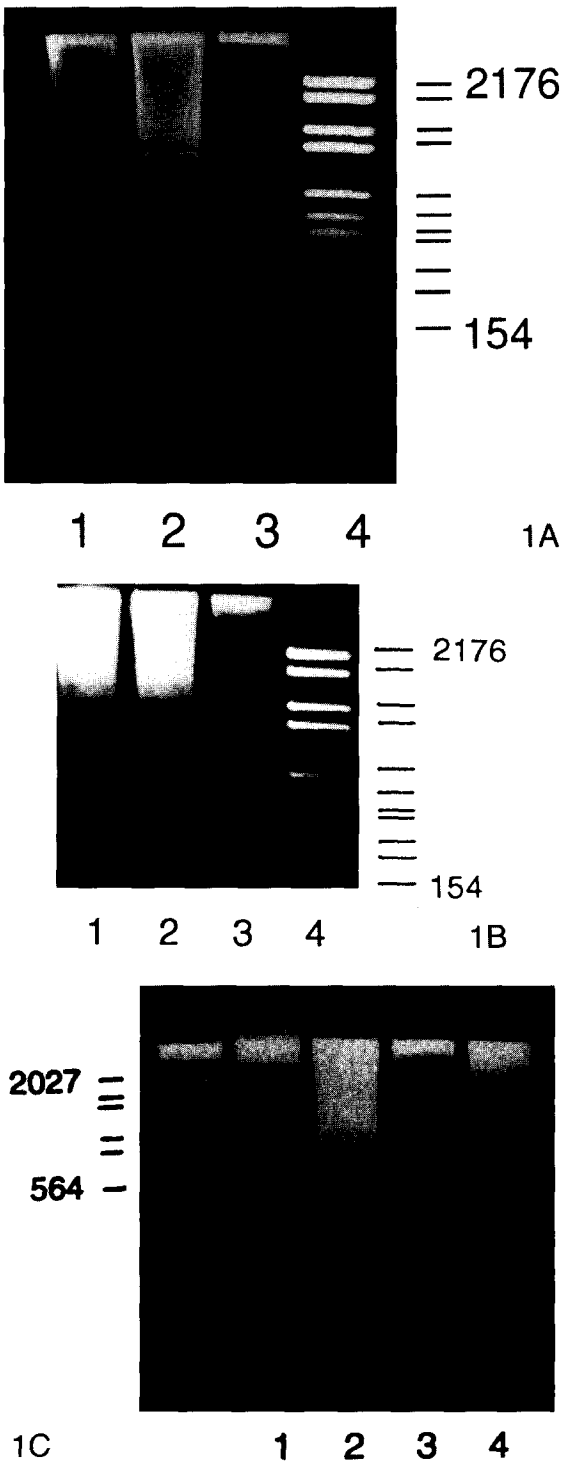

Fig. 1. (a) DNA fragmentation in mesangial cells induced by dibutyryl cyclic AMP. Cells were incubated for 24 h with dibutyryl cyclic AMP 5 mM (lane 1), 1 mM (lane 2) or vehicle (lane 3); lane 4 displays DNA markers. (b) DNA fragmentation in mesangial cells induced by forskolin. Cells were incubated for 24 h with forskolin 30 μM (lane 1), 10 μM (lane 2) or vehicle (control, lane 3); lane 4 displays DNA markers. (c) Induction of DNA fragmentation by salbutamol and inhibition of forskolin induced DNA fragmentation by cycloheximide. Mesangial cells were incubated for 24 h with vehicle (control, lane 1), forskolin 15 μM (lane 2), forskolin 15 μM plus cycloheximide 10 μM (lane 3) or with salbutamol 25 μM (lane 4).

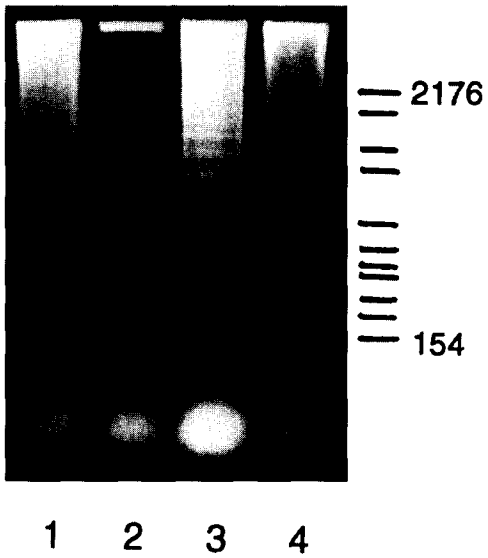

Fig. 2. Cyclic AMP induction of DNA fragmentation in mesangial cells is not mediated by nitric oxide. Mesangial cells were incubated for 24 h with forskolin 10 μM (lane 1), vehicle (control, lane 2), forskolin 10 μM plus L-NMMA 1 mM (lane 3) and with forskolin 10 μM plus L-NMMA 2 mM (lane 4).

4. Discussion

Elevated levels of intracellular cyclic AMP are known to induce apoptotic cell death in rat leukemia cell lines [14], in rat primary granulosa cells [15], in rat thymocytes [16] and in human B lymphocytes [17]. Here we extend these reports, substantiating that rat renal mesangial cells are susceptible to cyclic AMP-induced apoptosis.

The cellular cyclic AMP signalling pathway was activated by different means: (i) a receptor-coupled mechanism using the β_2-adrenergic receptor agonist salbutamol, (ii) direct stimulation of the catalytic subunit of adenylyl cyclase with forskolin or (iii) exposure of the cells to a membrane-permeant cyclic AMP analogue, dibutyryl cyclic AMP. Activation of the apoptotic programme was verified on a biochemical basis by detection of apoptotic DNA degradation (Fig. 1Fig. 2Fig. 3) and on the basis of morphology by demonstrating typical nuclear chromatin condensation in cyclic AMP-activated mesangial cells (Fig. 4). Cyclic AMP-dependent induction of apoptosis was found to be dependent on protein synthesis and independent of induction of nitric oxide production (Fig. 1c, Fig. 2). Whereas chromatin condensation was undetectable in control cells, approximately 10% of the cyclic AMP-activated mesangial cells showed an apoptotic morphology. We propose that only a small proportion of the whole cell population is susceptible to cyclic AMP-induced apoptosis at a certain time point. However, since mesangial cells have a prominent phagocytic capacity [3] the overall degree of apoptotic cell death may be underestimated because apoptotic cells are likely to be phagocytosed rapidly by their neighbours. The molecular mechanism of cyclic AMP-induced apoptosis is not known, but the intracellular transduction mechanism is believed to be exclusively protein kinase A mediated [18]. A fine-tuned crosstalk between the cyclic AMP/protein kinase A cascade and the mitogen-activated protein kinase family members has recently been suggested to control such fundamental processes as cell proliferation (for review see [19]) and apoptosis [20]. Two principal response patterns to cyclic AMP

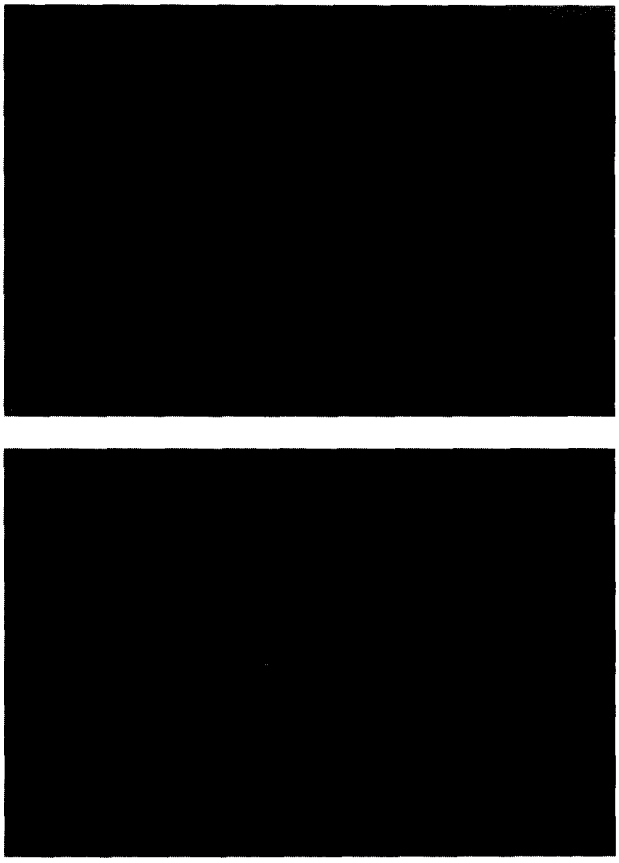

Fig. 3. In situ detection of cyclic AMP-mediated apoptosis in mesangial cells by TUNEL staining. Mesangial cells were incubated for 24 h (a) with vehicle (control) and (b) with forskolin (10 μM). Thereafter in situ detection of apoptotic cells by TUNEL staining was performed as described in section 2.5.

Table 1
Effects of cyclic AMP on cytosolic oligonucleosome-bound DNA in mesangial cells

Addition	DNA fragmentation
Control	100 ± 54.5
Salbutamol (25 μM)	398 ± 18.9*
Dibutyryl cyclic AMP (5 mM)	640.2 ± 79.6*
Forskolin (15 μM)	1009.3 ± 180**
Forskolin (15 μM) + cycloheximide (10 μM)	335.9 ± 33.1++
Cycloheximide (10 μM)	174.3 ± 88.5

Mesangial cells were incubated with vehicle (control) or the indicated compounds for 24 h. Cytosolic oligonucleosome-bound DNA was quantiated by an ELISA kit, DNA fragmentation is indicated as % of untreated control cells. Data are means ± S.D. of three independent experiments. Significant differences from control: $^{**}P < 0.01$; $^{*}P < 0.05$ (ANOVA). Significant difference from corresponding stimulation with forskolin alone: $^{++}P < 0.01$ (ANOVA).

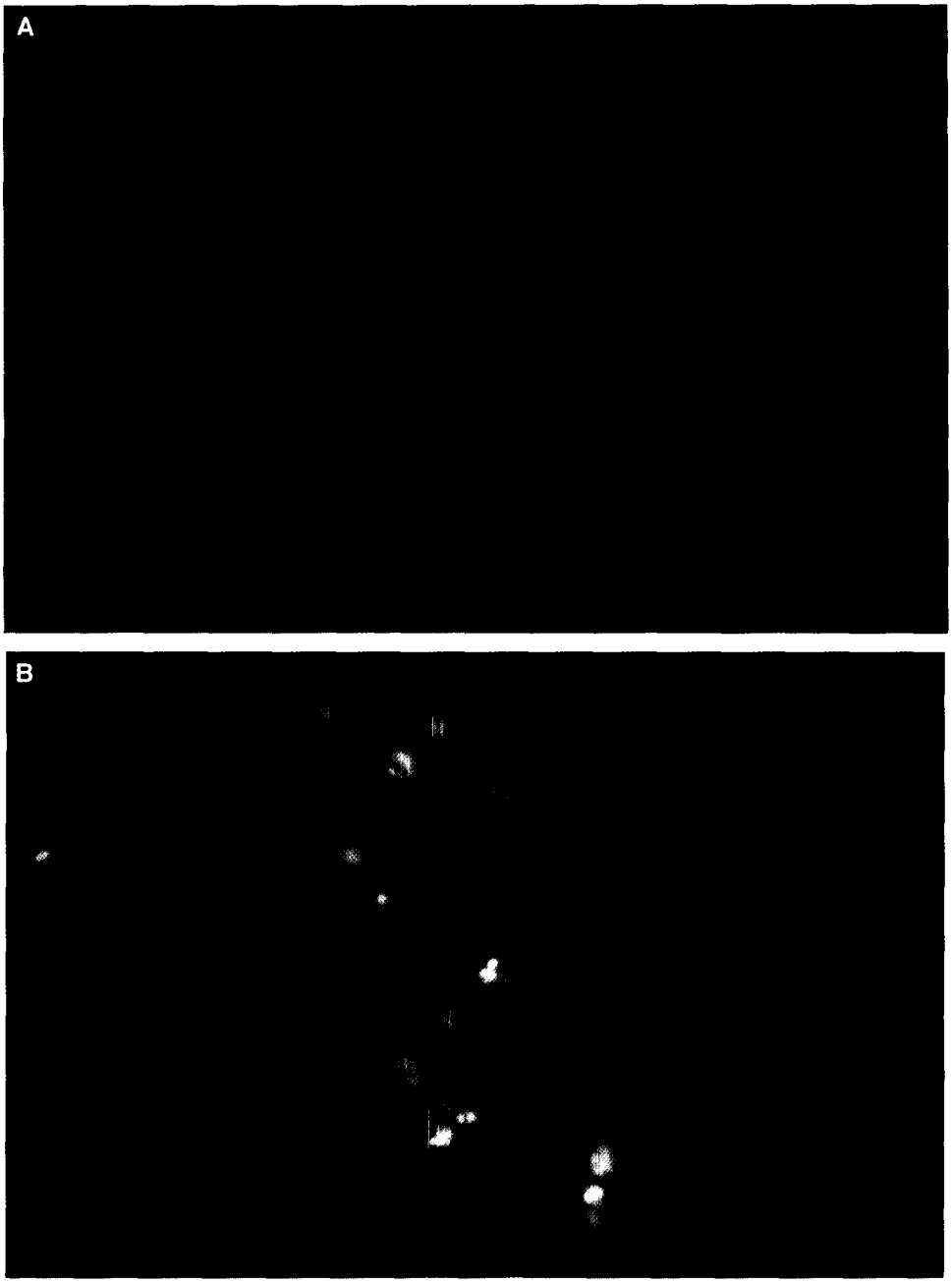

Fig. 4. Detection of cyclic AMP-induced nuclear chromatin condensation in mesangial cells. Mesangial cells were incubated for 24 h (a) with vehicle (control) and (b) with forskolin (20 μM). Thereafter the nuclei were stained with Hoe-33258 in order to detect chromatin condensation.

have been described. Depending on the cell type cyclic AMP either inhibits or activates extracellular signal regulated kinases and thus either blocks or stimulates cell proliferation by a mechanism which involves protein kinase A-mediated Raf-1 kinase phosphorylation [19]. In a complementary fashion cAMP has been reported to modulate apoptosis in either a positive [14–16] or negative way [20,21] by modulating the dynamic balance between two members of the mitogen-activated protein kinase family [20]. Importantly, cAMP has been shown to inhibit [^{3}H]thymidine incorporation into growth factor-activated mesangial cells [22] and attenuates extracelluar signal regulated kinases in response to angiotensin II or platelet-derived growth factor [19]. It is tempting to speculate that cAMP triggers an apoptotic signal in mesangial cells by unbalancing the opposing effects of specific members of mitogen-activated protein kinase modules in a way complementary to that observed in PC-12 cells [20]. Several mediators are known to act on glomerular mesangial cells by stimulating the cyclic AMP signalling pathway. Examples are histamine, adenosine, prostaglandin E_2, dopamine and others (for review see [3]). Recently Baker et al. [5] reported that mesangial cell apoptosis is induced during the course of Thy-1 nephritis in rats. In the pathogenesis of glomerular inflammatory diseases matrix production by mesangial cells and glomerular hypercellularity are supposed to be key steps in the progression towards sclerosis and loss of kidney function. We suggest that activation of the cyclic AMP signalling pathway during the course of glomerular inflammatory diseases may result in two beneficial effects complementing each other in counteracting glomerular hypercellularity: the inhibition of mesangial

cell proliferation and the simultaneous induction of apoptosis of surplus mesangial cells. This may guarantee a sophisticated orderly process of repair.

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