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GLUCOCORTICOIDS BLOCK OXIDANT-INDUCED ENDOTHELIAL CELL DEATH

Glucocorticoids are regarded as proapoptotic antiinflammatory glucocorticoids that are able to induce apoptosis of lymphoid cells. Our investigations in contrary have focused on the antiapoptotic properties of synthetic glucocorticoid Dexamethasone in endothelial cells. Endothelial cell line EAhy-926 was treated with dose range of hydrogen peroxide ranging from 10 mM to 500 mM with and without pretreatment with Dex. Cellular parameters such as viability, proliferation rate, lactate dehydrogenase release and mode of death were investigated. Results of experiments indicate that pretreatment with glucocorticoid Dexamethasone at 1 M for 24 hours reduces amount of cell death from 80-85 % at 250 mM and 95 % at 500 mM of hydrogen peroxide to 35 % and 57 % respectively. Lactate dehydrogenase release and fluorescent microscopy analysis with HOECHST 33342 and propidium iodide indicated that blockage in cell death occurs before mitochondrial damage and establishement of caspase cascade.

Introduction

Glucocorticoids are widely regarded as anti-inflammatory agents, capable of reducing immune activation. It is well-established fact that glucocorticoids are able to induce apoptosis in lymphocytes and regulate many processes on the cellular level [1]. These observations have lead to the dogmatic thinking that glucocorticoids, typified by the synthetic agent, dexamethasone (Dex), are pro-apoptotic. [1]. Recent studies in this field have suggested that this situation does not pertain for epithelial and endothelial cell types as well as some types of cancer [2-4]. Recently published work on pituitary tumor cells [4] suggests, that glucocorticoids block oxidant-induced necrotic cell death and in endothelial cells they block oxidant-induced apoptosis [4]. The latter finding may have considerable implications for both neonatal and adult respiratory distress syndrome and in the development of cardiovascular disease where cellular damage is caused by generation of oxidants [5].

Apoptosis is a form of programmed cell death that is governed by distinct molecular mechanism with many key events and activators. Apoptosis is more preferable type of cell death and, in comparison to necrosis, it is an active energy dependent process, which causes minimal disturbance to the surrounding tissues. As opposed to the cellular lysis associated with necrosis, apoptotic cells are recognized by the immune system and removed before inflammatory response is established.

Generally, apoptosis can be induced by DNA and mitochondria damage associated with ionizing radiation, oxidative stress, and various apoptosis inducing factors [5]. During the induction of apoptosis the distinct chain of events takes place that is usually triggered by loss of mitochondrial function and cytochrome C release. Subsequently this response activates caspase enzymes cascade, which cleaves intracellular components and DNA [6]. Our findings have indicated, that Dex is capable of reducing proliferation rate of endothelial cells and their sensitivity to the oxidative stress, effectively protecting them form apoptotic cell death. Endothelial cell line EAhy-926 was used to study regulation of apoptosis initiation events and influence of the glucocorticoid dexamethasone on the flow of this process. Findings and results obtained in the current experiments and regarded as important in the treatment of respiratory distress syndrome, cardiovascular disease, reperfusion injury and many forms of vascularized cancers.

Materials and methods

Cell culture

EAhy-926 was obtained from ATCCC stocks and was cultured in basic DMEM with 10 % heat inactivated fetal calf serum, 10 mM L-glutamine, penicillin/streptomycin. Cells were kept at absolute humidity in incubator with 5 % $C0_2$ in the atmosphere. Passaging was performed with Trypsin-EDTA. Cells were seeded in 75 cm^2 flasks and in 48-well plates during experiments at the concentration of 1-3 x 10^5 cells/cm^2. Prior to experiments, cultures were allowed to attach and establish exponential growth. All experiments were performed at around 30 % confluence, as determined by microscopic observation.

DNA electrophoresis

Treated cells were collected from the wells by repeated aspiration and brief centrifugation at 10000 g. Complete cell pellets were gently resuspended and lysed in 2X DNA loading buffer (ABgene ltd, UK) containing 10 mM SDS. Lysate containing DNA was loaded in to the 1,7 % Low electro-endo-osmosis agarose prepared in Tris borate EDTA buffer (National Diagnostics, UK). Reverse pulse field electrophoresis was performed at 2,5 V/cm with frequency of 0,33 Hz forward and 1 Hz reverse. Electrophoresis persisted for 12 hours at 4 °C. After electrophoretic separation, gel was stained with ethidium bromide and visualized under UV transilluminator. Microphotographs of the gel are shown as negatives.

Lactate dehydrogenase release test.

Supernatant was removed from treated cells and centrifuged at 3000 g fro 5 minuted in order to remove cell debris. Obtained supernatant was transferred to a 96-well plate (100 ml) and 10 ml of LDH testing kit reaction mixture (Roche-Diagnistics) was added and mixed. Reaction was incubated for 30 min at 37 °C and scanned in multiplate reader set at 420 nm. Results shown as relative units comparatively to the kits internal control.

Microscopy and fluorescent microscopy

Leica DMIL microscope with IMC optics was implemented for visual analysis of the cultured material and Canon EOS 300D was used as capture device. HOECHST 33342 membrane permeable fluorochrome was added directly to the growth media at 10 mg/ml concentration. In addition, membrane impermeable propidium iodide (PI) was also added to the same wells at the concentration 2 mg/ml to distinguish between two forms of cell death. During apoptosis, cell membrane remains intact, hence all DNA material is stained blue by the HOECHST 33342, while during necrotic cell death, cell membranes are permeable to PI and DNA stains red. For HOECHST 33342 excitation wavelength was set at 360 nm with barrier filter at 430 nm. For PI exitation wavelength was 480 nm and barrier filter set at 520 nm.

Results

Apoptosis in EAhy-926 cell line was induced by a 10 mM to 500 mM dose range of hydrogen peroxide. Cell viability, upon treatment with oxidants, decreased in dose dependent manner and was down to 20-25 % at the dose of 250 mM and down to 5 % at 500 mM after 24 hour exposure (Figure 1). In the same time pretreatment of endothelial cells with 1 μM Dex for 24 hours reduced sensitivity to oxidative stress and viability levels were 65 % and 43 % at 250 mM and 500 mM, respectively.

Lactate dehydrogenase release testing indicated that upon treatment with high doses of oxidants significant amount of enzyme activity could be detected (Figure 2). This corresponded to morphological appearance of endothelial cells that displayed extensive necrotic death, especially evident at

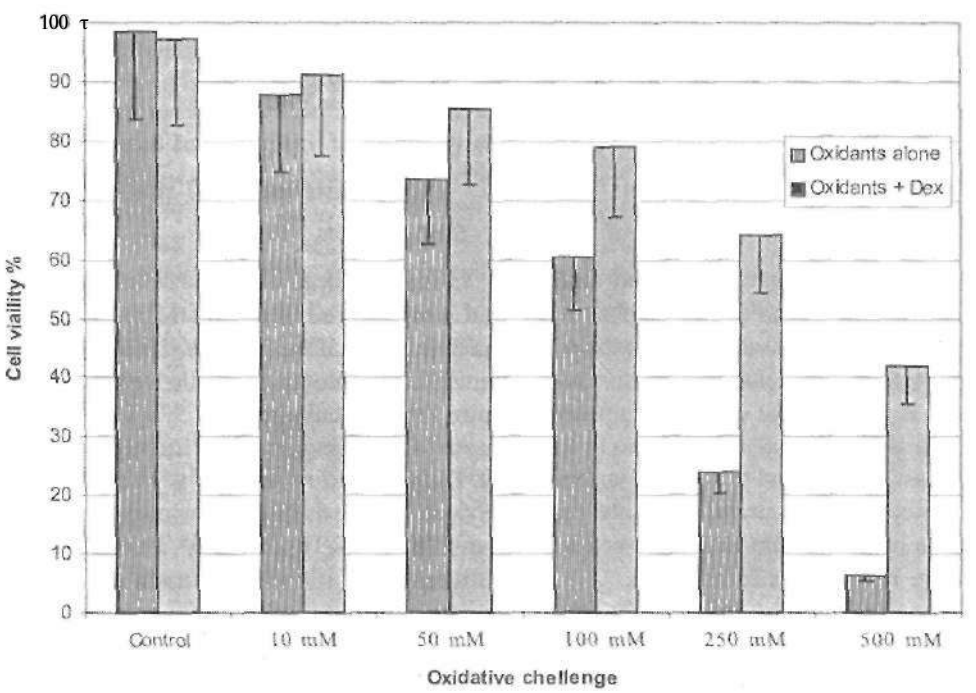

Fig. 1. Viability of EAhy-926 cell line upon treatment with the dose range of oxidants with and without 1 μM Dexamethasone pretreatment.

500 mM. At the same time very low LDH enzymatic activity was detected in the media of cultures exposed to Dex for 24 hours.

Microscopical analysis of the treated cells indicated that oxidants at **the dose** range of 100-250 mM were inducing apoptosis (Figure 3b), while higher doses induced classical necrosis (Figure 3c). At the same time pretreatment with Dexamethasone reduced sensitivity of Eahy-926 cells to oxidative challenge.

Fluorescent staining with HOECHST 33342 and PI indicated complete DNA fragmentation and formation of apoptotic bodies in some cells and loss of membrane integrity in others, depending on the dose of oxidative stress (Figure 4).

Patterns of DNA fragmentation associated with necrotic and apoptotic cell death were confirmed with reverse pulse field electrophoresis (Figure 5). Addition of Dex reduced DNA fragmentation associated with cell death giving some ideas about mechanisms of Dex protection. Lack of DNA fragmentation with Dex indicates that caspases are not activated and, therefore, Dex blockade takes place upstream of caspase cascade induction.

Discussion

Results of experiments indicated that 24 hour pretreatment with Dex reduced metabolic activity of the endothelial cell line and slowed down their proliferation rate up to 60 %. Furthermore, long-

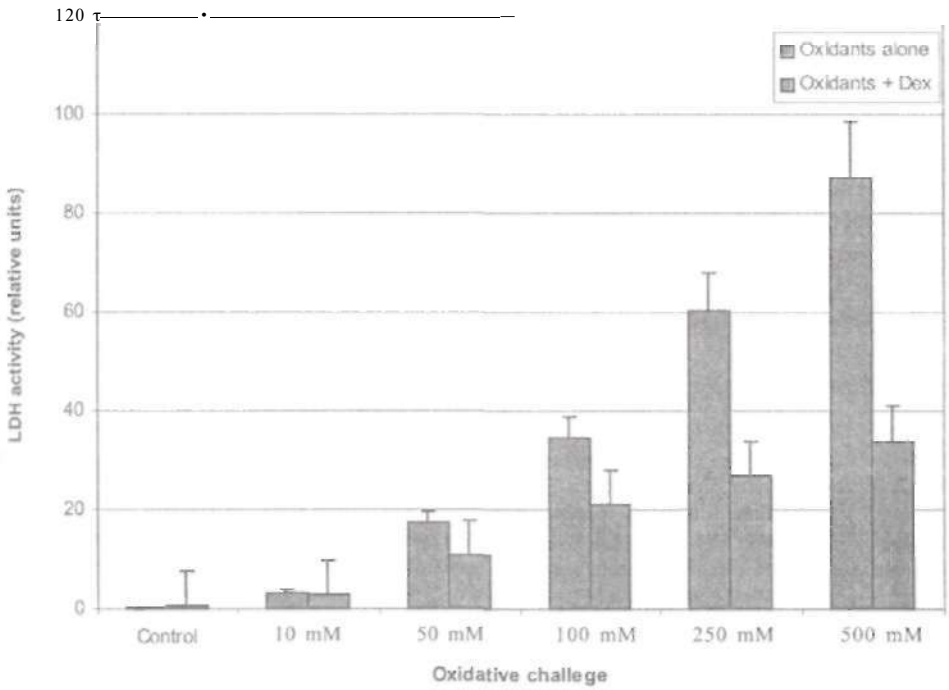

Fig. 2. LDH activity upon oxidative challenge with and without 1 μM Dex.

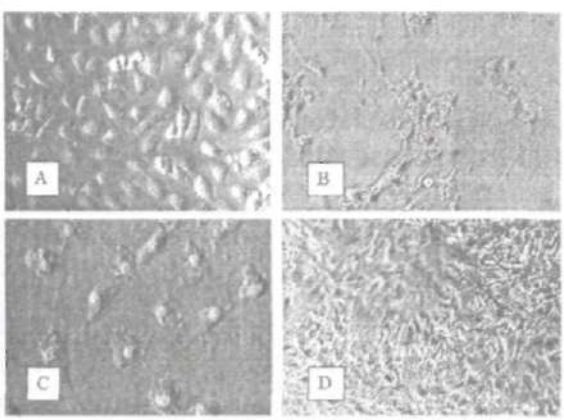

Fig. 3. Morphological appearance of EAhy-926 cell line in Control (A), characteristic apoptosis induced by 250 mM H_2O_2 (B), typical necrosis induced by 500 mM H_2O_2 (C) and death blockage at 250 mM triggered by preexposure to 1 μM Dex (D).

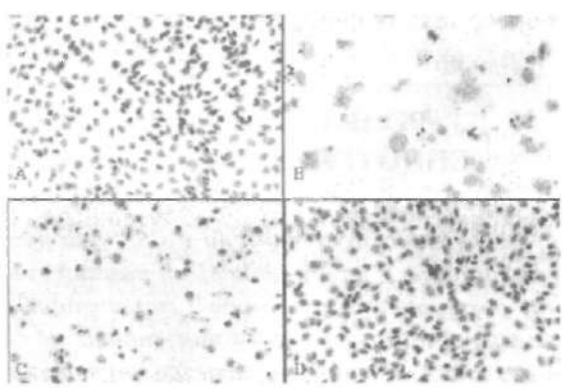

Fig. 4. Fluorescent microphotograph of EAhy-926 cell line undergoing apoptosis induced by 100–250 mM hydrogen peroxide (A), necrosis induced by 500 mM hydrogen peroxide (C) and prevention of cell death with Dexamethasone pretreatment (D) in comparison to control (A) (200X, negative monochrome fluorescent microphotograph).

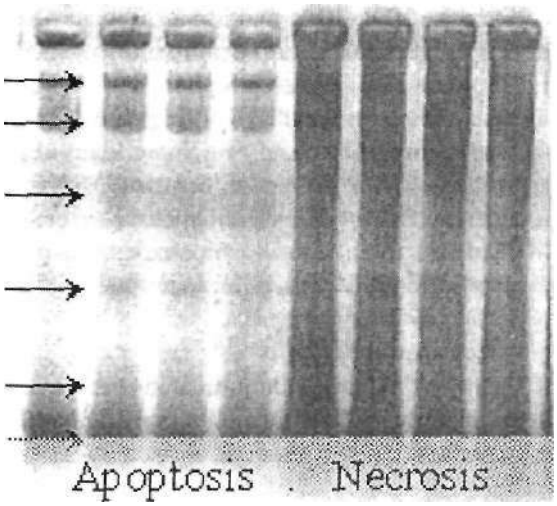

Fig. 5. DNA fragmentation patterns during apoptosis (indicated by arrows) and necrosis.

term exposure to Dexamethasone induced in vitro histogenesis, alignment of the cells into ordered bundles and reduced sensitivity of cultured cells to the lack of appropriate nutrients in the media. In this conditions cells completely stopped proliferation and could persist for duration of 30 and more days. Upon removal of Dexamethasone from the growth medium, cells rapidly (within 12-24) hours undergo death, mainly by necrosis. However, if cells were to be transferred to fresh medium containing Dex, they displayed high viability and regained their proliferative potential. Removal of Dexamethasone after initiation of proliferation did not induce cell death and even speeded up proliferation and sensitivity to apoptosis inducing agents.

Conclusions

Dex plays important role in the regulation of apoptosis acting as antiapoptotic agent in endothelial cells.

Effects of Dex are induced 24 hours after exposure, indicating that changes in cellular responses are dependent on major gene expression changes.

Intact DNA during apoptosis induction upon treatment with Dex indicates that effects of Dex are upstream of caspase cascade activation.

Cell-to-cell contacts and elements of histogenesis may play important role in the effect of Dexamethasone that is indicated by seizure of proliferation and alignment of the cultured endothelial cells upon reaching confluence.

These data suggest that Dex acts upstream of mitochondria and prevents activation of the caspase cascade.

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РОЛЬ ГЛЮКОКОРТИКОЇДІВ У БЛОКУВАННІ ЗАГИБЕЛІ ЕНДОТЕЛІАЛЬНИХ КЛІТИН, ІНДУКОВАНОЇ ОКСИДАНТАМИ

Глюкокортикоїдні гормони у клінічній практиці розглядаються як проапоптичні протизапальні засоби. У нашій роботі ми розглядаємо антиапоптичну дію синтетичного глюкокортикоїду дексаметазону на моделі ендотеліальної клітинної лінії Eahy-926. Культури клітин обробляли перекисом водню у концентрації від 10 mM до 500 mMу присутності 1 μ дексаметазону та без нього. Вивчали життєздатність клітин, рівень проліферації, виділення лактатдегідрогенази та форми клітинної загибелі. Результати експериментів свідчать про те, що дексаметазон зменшує рівень загибелі клітин від 80-85 % та 95 % при 250 mM та 500 mM перекису водню до 35 % та 57 % з дексаметазоном, відповідно. Виділення лактатдегідрогенази та флуоресцентні дослідження з використанням HOECHST 33342 та пропідіум йодиду свідчать про те, що дексаметазон блокує загибель на домітохондріальній стадії, що попереджує активацію каспазного каскаду та індукцію апоптозу.