Effect of Octreotide on Apoptosis-related Proteins in Rat Kupffer Cells: A Possible Anti-tumour Mechanism

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Abstract. Background: Octreotide may prolong survival in patients with hepatocellular carcinoma, through an as yet unidentified mechanism. Kupffer cells play a key role in antitumour activity. Kupffer cell apoptosis is of major importance for the maintenance of this antitumour activity. Materials and Methods: We studied the in vitro effects of octreotide in the RNA expression of apoptotic and antiapoptotic proteins in isolated rat Kupffer cells, before and after exposure to lipopolysaccharide (LPS). Apoptotic and antiapoptotic gene expression was assessed using a semi-quantitative multiplex RT-PCR measuring bax, bcl-xS, bcl-2 and bcl-xl. LICE (caspase-3) mRNA was used as a measure of apoptosis. Results: Unstimulated Kupffer cells exhibited increased proapoptotic gene expression in a time-dependent manner, paralleled by a similar increase of LICE. LPS stimulation decreased the expression of proapoptotic bax and bcl-xS mRNA without effecting the antiapoptotic proteins. A decrease of LICE expression became significant at 48 hours. Octreotide showed a reduction of proapoptotic proteins, accompanied by an early increase and a late reduction of antiapoptotic proteins and a net decrease of LICE expression. A combination of LPS and octreotide produced a similar effect with the exception of a late increase of LICE expression, probably caused by a late increase of bax and bcl-xS. Conclusion: LPS and octreotide reverse the observed increased apoptosis of cultured Kupffer cells by influencing both proapoptotic and antiapoptotic proteins. Therefore, the antitumour effect of octreotide in hepatocellular carcinoma may, in part, be explained by its antiapoptotic effect on Kupffer cells.

Kupffer cells (KC) account for approximately 15% of the total liver cell population in number and seem to play a central role in the homeostatic response to liver injury (1). These cells contribute to the formation of the sinusoidal lining with sinusoidal endothelial cells, stellate cells and pit cells (2). Among them the KC are the first cells of the mononuclear phagocyte system to be exposed to immunoreactive, infective, or toxic (e.g. ethanol) material absorbed from the gastrointestinal tract (3). Due to their key location, Kupffer cells might function as antigen-presenting cells and as scavengers of microorganisms, endotoxins, degenerated cells, immune complexes and various blood-borne particles (4).

Apoptosis, or programmed cell death, is of major importance for the maintenance of the liver tissue balance (6). In contrast to necrosis, apoptosis does not produce a harmful inflammatory response as it is controlled by numerous mechanisms including cell surface receptor interactions, cytokines and pro- and anti-apoptotic proteins of the Bcl-2 family (7). Kupffer cells exhibit vigorous phagocytic activity for apoptotic bodies (1) and it has been shown that they express specific receptors and ligands related to the apoptotic mechanism. It has been suggested that they might act as regulators of the programmed cell death of hepatocytes and T lymphocytes (8). A study of KC in co-culture with tumour cells, has recently established that, after LPS activation, they secrete proapoptotic Bax proteins capable of altering the balance between Bax and the antiapoptotic Bcl-2 proteins in favour of the former due to increased iNOS and TNF-α levels. More recently published observations have also implicated Kupffer cells in immunomodulation through apoptosis (9,10) and speculated that they could possibly express apoptosis related-genes as a consequence of LPS induction.

Somatostatin is a phylogenetically ancient, multigene family of peptides with two bioactive products: somatostatin-14 and somatostatin-28, acting both as neurotransmitter and neurohormone (11). In the periphery, somatostatin is secreted in the gastrointestinal tract and pancreas either from nerve endings in the intestinal mucosa and hepatoportal area or from non neuronal cells distributed throughout the length of the gastrointestinal tract (12). There is accumulating
evidence that somatostatin may have direct immuno-modulatory actions (13). Octreotide, the first synthetic somatostatin analogue introduced for clinical use, has been used for the treatment of secreting pituitary adenomas, metastatic islet-cell and carcinoid tumours, somatostatin receptor-positive neuroendocrine tumours and adeno-carcinomas (14,15). As this somatostatin analogue has been observed to act as a potent antitumour/antiproliferative compound in various studies (16,17), we have previously reported that octreotide treatment significantly improves survival and is a valuable alternative in the treatment of inoperable hepatocellular carcinoma (18,19). Kupffer cells may be implicated in the antitumour activity of octreotide in the liver (20).

In the present study, therefore, we examined the expression of apoptotic and antiapoptotic agents by rat Kupffer cells after exposure to LPS and octreotide, in an effort to explain the favourable results we observed from our clinical studies.

Materials and Methods

**Materials.** Hanks’ balanced salt solution (HBSS) and HBSS Ca++/Mg++-free were prepared from stock HBSS (10X, GibcoBRL, Paisley, UK) and supplemented with 4mM HEPES (GibcoBRL) and 0.07% sodium bicarbonate (GibcoBRL) (pH 7.4). Solutions for enzymatic tissue digestion with Pronase (Boehringer-Mannheim, Mannheim, Germany), DNAse (Boehringer-Mannheim) and Collagenase B (Boehringer-Mannheim) were freshly prepared in HBSS on the day of cell isolation. Gradient was prepared from Optiprep (Nycomed-Pharma, Oslo, Norway) appropriately mixed with HBSS. Dulbecco’s modified eagle medium (DMEM) (GibcoBRL) supplemented with 10% foetal bovine serum (FBS) (GibcoBRL) and 100 U/ml penicillin/streptomycin (GibcoBRL) was used for cell culture. LPS was from *Escherichia coli* (026:B6; phenol extract, Sigma-Aldrich, Steinheim, Germany). Octreotide (Sandostatin 0.1mg/ml) was purchased from Novartis (Basel, Switzerland).

**Kupffer cell isolation.** Kupffer cells were isolated from pathogen-free male Sprague-Dawley rats over 12 months old (450-600 g). Animals were fed food and water *ad libitum*. All studies were approved by the Veterinary Administration Office of Heraklion, Ministry of Agriculture, Greece, and conformed to the National and EU directions for the care and treatment of laboratory animals. Cell isolation was performed according to previously published methodology (21). Briefly, liver tissue was enzymatically digested by perfusion through the portal vein using a combination of 0.2% Pronase and 0.01% Collagenase in HBSS at 10 ml/min, 37°C in a non-recirculating fashion. Following a second incubation with 0.03% Pronase and 0.01% DNAse at 37°C for 30 min, the liver homogenate was filtered through a nylon mesh to remove undigested tissue and the cell suspension was loaded on a double layer discontinuous Iodixanol gradient of 11.7 and 17.6% Optiprep in order to separate sinusoidal cells from viable hepatocytes (1400g for 17 min at 4°C). Kupffer cells were further separated from the other sinusoidal cells by a modification of the centrifugal elutriation method originally described by Knook (22). HBSS was used as elutriation medium. Ten ml aliquots of the cell suspension were introduced into the elutriation system at a flow rate of 18.5 ml/min. After 200 ml of medium had passed through and a cell pellet was clearly formed in the elutriation chamber, the flow rate was increased stepwise from 18.5 to 25, 35, 45, 60, 80 and 100 ml/min. A volume of 100 ml of cell suspension was collected at each point. KC collected at flow rates of 45 and 60 ml/min at 2500 rpm and 25°C were further purified by the method of selective adherence to plastic. This final step resulted in a cultured population of >95% ED-2-positive (antibody specific for liver Kupffer cells), >95% exhibiting non-specific esterase activity, which is also characteristic for Kupffer cells, tested as previously described (21,23). Viability was more than 98% by trypan blue exclusion test.

**Cell culture.** The cells were seeded on 6-well plates at a density of 1-3X10⁶/well and cultured in DMEM (GibcoBRL) supplemented with 100 U/ml penicillin/streptomycin (GibcoBRL) and 10% FBS (GibcoBRL). For experiments, cultured cells 24 hours after isolation were washed and cultured in fresh medium without foetal bovine serum 4 hours before stimulation. Growth-arrested cells were treated with the appropriate concentrations of stimuli in medium and incubated as follows. In order to evaluate LPS and...
octreotide-mediated apoptosis, cultured KC were incubated with 1µg/ml of LPS or 1 ng/ml of octreotide up to 48 hours under serum-free conditions. This concentration of octreotide had been found to maximally affect isolated Kupffer cells in our previous experiments (21). For the evaluation of the effect of octreotide on LPS-stimulated KC, cells cultured in the presence of 1µg/ml of LPS were incubated with 1 ng/ml octreotide up to 48 hours. Cultured rat Kupffer cell monolayers were harvested at 6-, 12-, 24- and 48-hour intervals by using 1ml/well of Trizol™ LS Reagent (Invitrogen, Carlsbad, California, USA) and immediately stored at -70°C for later quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis.

Figure 2. Densitometry analysis for PCR products of bax mRNA expression by Kupffer cells, measured as densitometry readings of PCR products adjusted to the housekeeping gene (GAPDH) in arbitrary units. Results are expressed as mean ± SEM and are representative of four experiments. Significance is indicated by * for p<0.05 and ** for p<0.01 compared to the initial expression at 6 hours.
Multiplex RT-PCR detection of rat apoptosis gene expression. Total RNA was extracted by the Trizol LS Reagent homogenates according to the manufacturer’s instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm. One microgram of total RNA was reverse transcribed as previously described (24). The resultant cDNA was amplified using sense and antisense primers for the expression of apoptosis-related genes. A commercially available kit was used (Rat Apoptosis Set 2 – Quantitative Multiplex PCR®) (rAPO2G-Quantitative Multiplex PCR® kit, Biosource, Camarillo, California, USA) which allows the simultaneous amplification of specific ORF regions of the rat bax, bcl-xS (proapoptotic), bcl-2, bcl-xL (antiapoptotic) and LICE (caspase-3) set of genes; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was

Figure 3. Densitometry analysis of bcl-xS mRNA expression by Kupffer cells.
included in the gene set as an internal standard. The kit also included a positive control, which was simultaneously amplified for every assay and was used as an indicator. The temperature profile of the PCRs consisted of a melting step at 96°C for 1 minute (hot start) followed by 2 cycles of denaturing step at 96°C for 1 minute and an annealing step at 59°C for 4 minutes; the procedure was followed by 33 cycles of 1 minute at 94°C, 2.5 minutes at 59°C, with a final extension step of 10 minutes at 70°C. Independent experiments established that 33 cycles were within the linear range of the multiplex PCR assay as the manufacturer indicated. PCR products were separated on a 2% agarose gel (Agarose PCR Grade, Sigma-Aldrich, Steinheim, Germany) and stained with ethidium bromide (Sigma-Aldrich). Images were examined with a UV transilluminator, photographed using the GelDoc 2000.

Figure 4. Densitometry analysis for PCR products of LICE (caspase-3) mRNA expression by Kupffer cells.
Imaging Apparatus (BioRad, California, USA), analyzed using Quantiscan™ (Version 1.5, Biosoft, UK) analysis software and the relative band intensities were calculated. A ratio of the densitometric value of each RNA over the value of the housekeeping gene (GAPDH) was constructed. All ratios were the mean of 4 consecutive experiments and results are expressed as mean ± SEM. LICE (Caspase 3) mRNA was used as an index of apoptosis.

Statistical analysis. The value at 6 hours was considered as the basic ratio and the others were compared to it by the Student’s t-test for unequal variances with Yates correction for small numbers.
Results

Isolated Kupffer cells from four rats were used in four sets of identical experiments. Kupffer cells from all animals showed similar results in response to stimulation with both LPS and octreotide. Growth arrested unstimulated Kupffer cells (Figure 1) were found to express proapoptotic genes. The expression of both bax and bcl-xS was increased in a time-dependent manner up to 48 hours (Figures 2a and 3a). These findings were paralleled by a time-dependent

Figure 6. Densitometry analysis for PCR products of bcl-xL mRNA expression by Kupffer cells.
increase of caspase-3 (Figure 4a). Interestingly, at 48 hours, Kupffer cells expressed for the first time the antiapoptotic gene bcl-2 (Figure 5a).

Stimulation with LPS induced a time-dependent reduction of the expression of bax and bel-xS mRNA at 12, 24 and 48 hours (Figures 2b and 3b, respectively). The antiapoptotic bcl-xL expression remained unchanged (Figure 6b), while bcl-2 disappeared at 48 hours (Figure 5b). LICE (caspase-3) also showed a time-dependent decrease (Figure 4b).

Stimulation with octreotide had a differential effect on the expression of apoptosis-related proteins by Kupffer cells depending on the duration of the exposure. In detail, the time course study has shown that stimulation with octreotide showed a time-dependent reduction of proapoptotic bax and bel-xS (Figures 2c and 3c), accompanied by a similar reduction in caspase-3 (Figure 4c), a delayed (24 and 48 hours) reduction of bel-xL (Figure 6c) and an early induction (6 and 12 hours) of bcl-2 (Figure 5c).

Cultures of KC stimulated with a combination of LPS and octreotide showed a reverse of the action of octreotide on the late reduction of bel-xL and also of bcl-2 at 12 hours (Figures 6d and 5d). Compared with octreotide alone, there was a similar reduction of the proapoptotic bax (Figure 2d) and bel-xS (Figure 3d) with the notable exception of a late increase at 48 hours for both. Similarly caspase-3 was reduced at 6, 12 and 24 hours and increased at 48 hours (Figure 4d).

Discussion

Kupffer cells, the resident liver macrophages, play an important role in the regulation of the liver immune system and actively participate in the defence against viruses and tumour cells. Kupffer cell activation has been proposed as a possible mechanism of liver antitumour activity (25).

Early experimental evidence has shown that octreotide, a synthetic analogue of natural somatostatin, inhibits fibrosarcoma and adenocarcinoma liver metastases (26,27). These early observations were confirmed in further experimental models, including nitrosomorpholine-induced hepatocarcinogenesis (28) and implantation of Morris hepatoma 3924A cells in rat livers (29). We have previously reported that octreotide significantly improved survival of patients with hepatocellular carcinoma (18,19). However, the mode of action of octreotide remains unclear. In an interesting study, nude mice were xenografted with a neuroendocrine cell line and received treatment with either octreotide or interferon-α (30). A 3-fold increase of apoptotic cells was observed in the octreotide group but not in the interferon-α group. Octreotide was reported to increase the activity of the Kupffer cells and this effect was paralleled by a significant reduction of growth in experimental liver tumours (20,31). It seems, therefore, that any effect on apoptosis of Kupffer cells would influence such an antitumour activity. In the present study, we investigated the expression of apoptosis-related proteins in isolated rat Kupffer cells cultured with LPS and octreotide. As an index of apoptosis, we used the expression of caspase-3 (LICE for rats), a key enzyme in the regulation of the apoptotic process.

Our results have shown that unstimulated cells expressed RNA of the proapoptotic proteins bax and bel-xS and that expression was gradually increased in a time-dependent manner. This observation was to be expected due to the progressive apoptosis that would naturally occur in our culture system. Moreover this increase was paralleled by a similar time-dependent increase in LICE. Interestingly, after a 48-hour culture, the antiapoptotic gene bcl-2 was for the first time induced and this might indicate that cultured Kupffer cells may in fact block further apoptosis by producing antiapoptotic monokines. This point requires further research.

Stimulation of Kupffer cells by lipopolysaccharide leads to decreased expression of the proapoptotic proteins bax and bel-xS RNA and this is possibly the explanation of the observed increased activity of Kupffer cells after LPS stimulation that we and others have previously reported (6,10,21). The anti-apoptotic protein bcl-2 that appeared after 48 hours of culture of unstimulated Kupffer cells was not present after LPS stimulation. In accordance with the above findings, LICE expression was slightly reduced at 6, 12 and 24 hours and more so at 48 hours, indicating that LPS stimulation possibly reduces apoptosis of Kupffer cells.

The most interesting finding of our study, however, was the observed effect of octreotide on the expression of apoptosis-related proteins. Early incubation (at 6 and 12 hours) led to an induction of the antiapoptotic protein bcl-2 while protracted incubation led to a suppression of both bcl-2 and bel-xL. By contrast, the RNA expression of the proapoptotic proteins bax and bel-xS showed a time-dependent constant decrease. A similar time-dependent reduction of LICE RNA expression was also noted after stimulation with octreotide. It may well be that the reported increased activity of Kupffer cells in experimental liver tumours depicts this antiapoptotic effect.

Finally, the results from the concomitant incubation of Kupffer cells with both octreotide and LPS show a similar reduction of LICE RNA expression at 6, 12 and 24 hours. Antia apoptotic protein bel-xL RNA expression remained constantly high at all time intervals which may indicate that LPS acts synergistically, at least initially, in inhibiting apoptosis with octreotide. However, in contrast to either LPS stimulation alone or octreotide incubation, at 48 hours there is a rise of LICE RNA expression. This is possibly due to the observed increased expression of both bax and bel-xS at 48 hours. In clinical terms, this might suggest that in the presence of endotoxaemia (a common finding in patients with terminal stage liver cirrhosis with hepatocellular carcinoma), the antitumour effect of octreotide may be
blunted when endotoxinaemia is protracted. However, more studies are required to clarify this situation.

In summary, octreotide causes a time-dependent decrease of the expression of proapoptotic proteins in rat Kupffer cells, accompanied by an early induction of antiapoptotic proteins. These results may, in part, explain the beneficial effect that this drug exerts in both experimental liver tumours and hepatocellular cancer patients.

References


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