Inhibition of puromycin-induced apoptosis in breast cancer cells by IGF-I occurs simultaneously with increased protein synthesis*

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The objective of the following work was to study the apoptosis inducing effect of puromycin in MCF-7 breast cancer cells and compare this effect with cycloheximide and emetine, 2 other inhibitors of protein synthesis. We also wished to investigate if the apoptosis modulating effect of insulin-like growth factor-1 (IGF-I) was similar for the 3 inhibitors.

An immunological assay, quantifying mono- and oligonucleosome fragments and morphological criteria after nuclear staining, were used to study apoptosis. Protein synthesis was measured by incorporation of ^{3}H-leucine in the cells, and solution hybridization and Western blot were performed to estimate IGF-I receptor m-RNA and IGF-I receptor protein respectively.

Puromycin at 0.5 μg/ml induced a high level of apoptosis in MCF-7 breast cancer cells, although there was still a non-negligible amount of synthesized protein. In the case of cycloheximide and emetine, apoptosis occured when protein synthesis was almost completely blocked. IGF-I at a concentration of 10 ng/ml significantly reduced the level of apoptosis induced by puromycin, emetine, or cycloheximide. We also noticed a parallel increase in ^{3}H-leucine incorporation when apoptosis induced by puromycin was lowered as an effect of IGF-I, in contrast to cycloheximide and emetine where IGF-I reduced the apoptosis level without increasing the ^{3}H-leucine incorporation. At a higher concentration of puromycin (5.7 μg/ml), which blocked protein synthesis, IGF-I at 10 ng/ml did not reduce apoptosis. The level of IGF-I receptor m-RNA was not influenced by the use of a concentration of puromycin (0.5 μg/ml) inducing a high degree of apoptosis.

These results suggest, that reduction of puromycin-induced apoptosis by IGF-I occurs simultaneously with increased protein synthesis, in contrast to emetine and cycloheximide. Furthermore it would appear that puromycin-induced apoptosis is not caused by reduced levels of IGF-I receptors.

Key words: growth factors, cycloheximide, emetine, puromycin

Apoptosis is a cell death process, which involves a series of well-organized events, requiring active cell participation and is vital during tissue differentiation and remodelling [3, 19, 39]. Furthermore apoptotic cell death is an important feature in tumor growth regulation [24]. Cancer treatment with chemotherapy and γ-irradiation kills target cells, primarily by inducing apoptosis [20]. Apoptotic cell death is normally executed through the cascade-like activation of caspases, which are intracellular cysteine proteases. The action of the caspases results in morphologic apoptotic characteristics, including chromatin condensation, DNA fragmentation, nuclear shrinkage and formation of apoptotic bodies, which are phagocytosed by neighbouring cells [3, 19]. The addition of IGF-I and other different growth factors can abolish or reduce cell death in different systems [2, 14, 21, 46]. IGF-I, which is a polypeptide hormone, binds to its transmembrane receptor on the surface of target cells [12]. This event is followed by an activation of intracellular signaling pathways, especially those involved in mitogenesis and differentiation [12].

On a number of occassions apoptosis has been shown to be suppressed by inhibitors of messenger RNA or protein synthesis, such as actinomycin D and cycloheximide respectively [7, 40]. In other situations these inhibitors have no blocking effect; Specific examples include apoptosis of target cells induced by cytotoxic T-lymphocytes [11], apoptosis

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of macrophages induced by gliotoxin [48], apoptosis in tumor cell lines induced by mild hyperthermia [45], and apoptosis caused by the absence of integrin-mediated signals [47]. However, puromycin and other inhibitors of protein synthesis have been shown to induce apoptosis in some normal and neoplastic cell populations [15, 30, 31, 42]. Furthermore the rate of protein synthesis is rapidly downregulated in mammalian cells following the induction of apoptosis in different ways [6]. In this work we examined induction of apoptosis in MCF-7 breast cancer cells by puromycin, a potent inhibitor of protein synthesis, and compared its effect with 2 other inhibitors of protein synthesis, emetine, and cycloheximide, each one inhibiting macromolecular synthesis via separate and distinct mechanisms [1, 18].

Material and methods

Material. Puromycin, cycloheximide, emetine, pepsin, and DAPI solution were purchased from Sigma Chemical Co. (St Louis, Mo, USA). Rnase, and proteinase K were purchased from Roche diagnostics scandinavia AB (Stockholm, Sweden). IGF-I was donated by Kabi/Pharmacia (Stockholm, Sweden), and ^{3}H-leucine, was purchased from Amersham International (Amersham, UK).

Cellculture. MCF-7 breast cancer cells were obtained from American type culture collection (Rockville, MD, USA). The cells were maintained as monolayers at 37 °C in an atmosphere containing 5% (vol/vol) CO_2 and cultured in Dulbeccos modified Eagles medium (DMEM), supplemented with 10% heat inactivated fetal calf serum (FCS), 200 IU/ml of penicillin, and 200 μg/ml of streptomycin. The cells were grown in flat bottomed plastic vials and passaged once a week. Chemicals, solutions, and the equipment used for cell cultures were purchased from GIBCO BRL Life technologies (Täby, Sweden), unless otherwise stated.

Apoptosis quantifying method. Cells were seeded in a 24-well tissue culture plate at a density of 40000 cells/well in 1 ml of ordinary culture medium. After 3 days of incubation, the culture medium was discarded, and the cells were incubated for 24 h with culture medium containing no serum. At the end of the incubation period, the culture medium was poured off and new medium containing IGF-I, insulin, and inhibitors of protein synthesis in different combinations was added for another 48 h as indicated for each experiment. When the last incubation period was completed, the incubation medium was saved and centrifuged in order to sediment any floating cells in the medium. The remaining cells in the bottom of each well were scraped off with a plastic device. All of the MCF-7 cells were combined into one sample. We used a cell death detection Elisa kit (Roche diagnostics scandinavia AB, Stockholm, Sweden) to quantify apoptosis [52]. Briefly, in the first incubation step, anti-histone antibodies were fixed adsorptively on the wall of a microtiter plate module. During the second incubation step, the nucleosomes contained in the sample became bound via their histone components to the immobilized anti-histone antibody. In the third incubation step, anti-DNA-antibodyperoxidase reacted with the DNA-part of the nucleosome. The amount of peroxidase retained in the immunocomplex was determined photometrically at a wave length of 405 nm with an ELISA plate reader (Anthos II; Anthos Labtec Instruments, Salzburg, Austria) with 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) as a substrate.

The annexin V/propidium iodide method for apoptosis detection, where annexin V binds to phosphatidylserine on the external surface of apoptotic cells, is considered to be reliable and registers apoptosis reatively early [49]. However, according to some authors, this method detects only a minority of apoptotic cells [49], and is considered as not suitable for quantification/semiquantification of apoptotic cells. Another way to detect apoptosis is by visualization by agarose gels of the characteristic DNA laddering of oligonucleosomal fragments [51]. This analysis requires the extraction of DNA from a large number of cells and can not be used to quantitate apoptosis.

Apoptotic cells are smaller in size than normal cells due partly to nuclear condensation. They can therefore after fixation and propidium iodide staining be quantitatively detected as hypodiploid cells in flow cytometric histograms where they appear as a peak to the left of the G0/G1 peak [34]. The TUNEL (Terminal dUTP Nick End-Labeling) technique detects cell nuclei containing fragmented DNA. This assay is well accepted and widely used for detection of apoptotic cells. However, TUNEL staining can not always distinguish apoptotic and necrotic cells [41].

Measurement of ^{3}H-leucin incorporation. Cells were cultured as described earlier. Two hours before the end of the 48 h incubation period with different reagents present, 1 μCi/ml of ^{3}H-leucine was added to each well. At the end of the incubation period, the cell culture medium was saved. The cell monolayer was washed once with 1 ml of 4 °C phosphate buffered saline (PBS) (PH 7.3) and three times with 4 °C 5% trichloroacetic acid (TCA). All washing fluids were added to the saved culture medium and in the fluid remaining cells were sedimented by centrifugation. The cell pellet was treated with PBS and TCA as described earlier and a solution containing 5% sodium dodecyl sulfate, 20 mM Na_2Co_3, and 2 mM ethylenediaminetetraacetat (EDTA) was added. The lysed cells were transferred to a scintillation vial together with 10 ml of scintillation fluid (Ultima Gold; Packard Instrument Company, Meriden, Conn., USA) for subsequent counting in a beta scintillation counter (1214 Rackbeta; LKB Wallac, Åbo, Finland).

DAPI-staining of MCF-7 cells. Cells were grown in plastic Petri dishes with a diameter of 35 mm (Labdesign, Stock-

holm, Sweden), until a confluent monolayer was established. The culture medium was then discarded and the cells were incubated for 24 hours with medium containing no serum, whereafter new medium, containing puromycin at 0.5 μg/ml, IGF-I at 10 ng/ml, or a combination of puromycin at 0.5 μg/ml and IGF-I at 10 ng/ml, was added for another 48 h. After this last incubation period the medium was poured off. The cells were fixed with 4% formaldehyde, washed twice with PBS (PH 7.4) and stained with DAPI (diamidino-2-phenylindole) 50 nM for 10 minutes. The specimen was mounted with Vectashield with DAPI (Immunkemi, Stockholm, Sweden) and the cells were examined by fluorescence microscopy (Nikon Eclipse E 800; Bergström Instruments, Stockholm, Sweden) at a magnification of 400x.

Quantitation of IGF-I receptor m-RNA. Cells were seeded in ordinary medium in Petri dishes with a diameter of 100 mm until confluence was established. The cells were then incubated for 24 h in serum free medium, whereafter incubation with puromycin at defined times and concentrations was initiated. Total nucleic acids (TNA) were prepared from cell cultures according to the method of DURNAM and PALMITER [13]. Briefly, floating cells were harvested from medium by centrifugation for 5 min at 3000 rpm (Beckman spinchrome R; Instrument AB, Bromma, Sweden) and solubilized together with the remaining cells in the Petri dishes in 4 ml of sodium dodecyl sulfate (SDS) containing buffer and homogenized with a polytron PT 1020 350 D (Kinematica GmbH, Lucerne, Switzerland). The samples were digested with proteinase K and extracted with phenol and chloroform. The TNA were then precipitated with 2 volumes of 70% ethanol. The total DNA concentration was measured by fluorometry according to the method of LABARCA and PAIGEN [26]. The concentration of mRNA was determined using [^{35}S]-UTP labeled RNA probes. The human IGF-I receptor probe was complementary to a 379 bp fragment coding for part of the α-subunit of the receptor [35]. The probes were prepared according to the method of MELTON et al [32], and were hybridized to TNA samples at 70 °C for 16–18 hours. Hybridization was performed in 40 μl of 0.6 M NaCl, 20 mM Tris-HCI (PH 7.5), 4 mM EDTA, 0.1% (w/v) SDS, 7.5 mM dithiothreitol (DTT), 25% (v/v) formamide; 10000 cpm of [^{35}S]-UTP labeled probes were used per incubation. The samples were exposed to Rnases, the hybrids were subsequently precipitated with 100 μl 6M trichloroacetic acid and collected on glass microfibre filters. Radioactivity was measured in a liquid scintillation counter (1214 Rackbeta; LKB Wallac, Åbo, Finland) and compared to standard curves constructed from synthetic RNA standards, which were complementary to the probes.

IGF-I receptor protein. MCF-7 cells were grown in 75 cm^2 vials (Corning Labdesign, Täby, Sweden) until a confluent monolayer was established. The cells were then incubated for 24 h with serum free medium. At the end of the incubation period the culture medium was poured off and serum free medium with or without puromycin at 139 μg/ml was added for another 24 h. After this last incubation period, loosened cells in the culture medium were sedimented by centrifugation and analyzed separately from adherent cells. Lysis buffer containing 20 mM Tris-HCL (PH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxychelate, 0.5–1% Triton X-100, 1.5 μg aprotinin/ml, 1.5 μg leupeptin/ml and 1 mM phenylmethylsulfonylfluorid was added to the cell fractions. Two and a half μl of a rabbit polyclonal IGF-I receptor β-subunit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added to cell lysate containing 1 mg protein. After 2 h incubation at 4 °C 50 μl protein A Sepharose (Pharmacia-Upjohn, Uppsala, Sweden) in 0.1% bovine serum albumin in lysis buffer was added. The mixture was kept over night at 4 °C during gentle rocking. The immunoprecipitate was washed 3 times with cold lysis buffer. The pellet was dissolved in 50 μl reducing buffer [27], whereafter the solution was boiled for 3 minutes. Twenty-five μl of this solution was put on a SDS-7.5% polyacrylamid gel electrophoresis and then transfered to a polyvinyl-difluoride membrane. The membrane was probed with the same polyclonal IGF-I receptor antibody (primary antibody) as described earlier. As a secondary antibody we used an anti rabbit antibody coupled with peroxidase. Detection was carried out with the ECL system (Amersham Pharmacia Biotech AB, Uppsala, Sweden). If the primary antibody was excluded in the Western blot analysis no protein band corresponding to the IGF-I receptor level could be detected.

Statistical analysis. Statistical calculations were performed using the unpaired two-tailed Student's t-test, or with one-way analysis of variance (ANOVA); p values <0.05 were considered significant.

Results

Effect of cycloheximide and emetine on induction of apoptosis with or without IGF-I. MCF-7 breast cancer cells were treated with cycloheximide at 50 μg/ml and emetine at 2 μg/ml respectively. After 48 h incubation, both inhibitors induced an apoptosis level significantly above the control value (Fig. 1A). If IGF-I was present, together with one of the inhibitors during the 48 h incubation period, the apoptosis levels were reduced to approximately the control value (Fig. 1A).

Effect of IGF-I on ^{3}H-leucine incorporation in the prescence of emetine or cycloheximide. MCF-7 brest cancer cells were treated with cycloheximide at 50 μg/ml and emetine at 2 μg/ml for 48 h. Two hours prior to end of incubation, ^{3}H-leucine was added to the cells. The ^{3}H-leucine incorporation was reduced to about 8% of the control value after

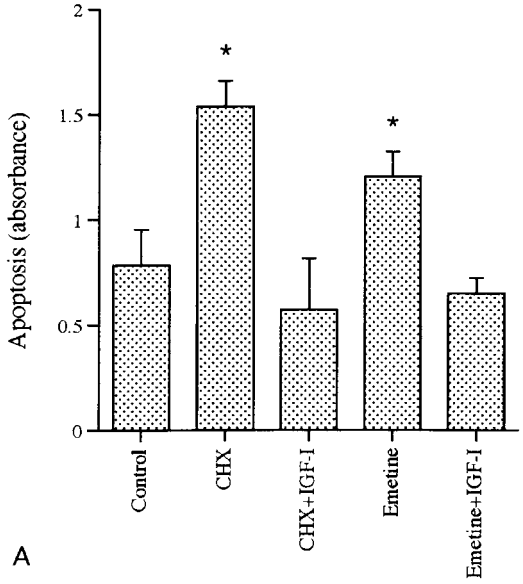

Figure 1A. Quantitation of apoptosis in MCF-7 cells after treatment with cycloheximide (CHX), emetine, and IGF-I. MCF-7 cells were incubated for 2 or 3 days with ordinary culture medium, and otherwise handled as described in the materials and methods section. CHX (50 μg/ml), emetine (2 μg/ml), and IGF-I (10 ng/ml) were present during the last 48 h of incubation, whereafter apoptosis was quantified as described in the materials and methods section. Each value represents the mean +SD of triplicate wells; *p at least <0.05 in comparison with control; Student's t-test. The experiment was repeated once with similar results.

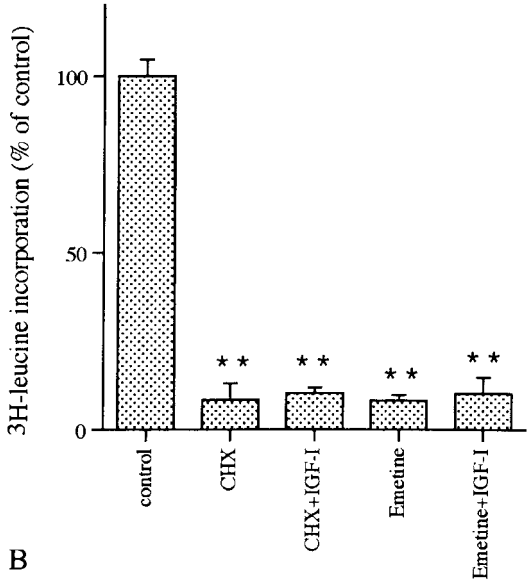

Figure 1B. Effect of cycloheximide (CHX), emetine, and IGF-I on ^{3}H-leucine incorporation in MCF-7 cells. The cells were handled as described in the materials and methods section. CHX (50 μg/ml), emetine (2 μg/ml), and IGF-I (10 ng/ml) were present during the last 48 h of incubation. Two hours before the end of incubation 1 μCi/ml of ^{3}H-leucine was added to each well. Each value represents the mean +SD of triplicate wells of 1 of 2 representative experiments. The mean control value corresponds to 4047 cpm; **p <0.001 in comparison with control; Student's t-test.

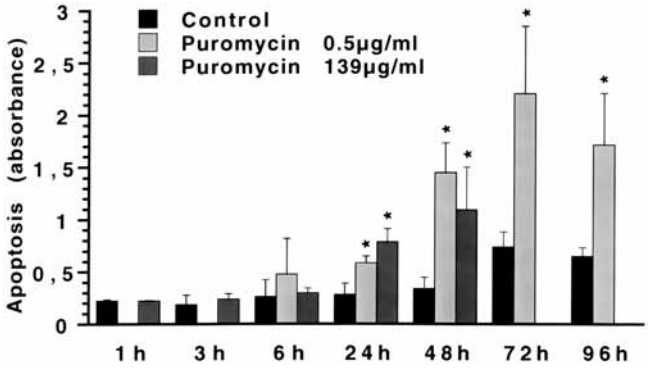

Figure 2. Kinetics of apoptosis in MCF-7 cells after puromycin treatment. MCF-7 cells were handled as described in the materials and methods section. Puromycin (μg/ml) was present at the indicated times, and concentrations. Each value represents the mean +SD of 2 or 3 different experiments, each one performed with duplicate or triplicate wells; *p at least <0.05 in comparison with the control value at the indicated times; Student's t-test.

treatment with only cycloheximide or emetine (Fig. 1B). If cycloheximide or emetine was combined with IGF-I at 10 ng/ml, the protein synthesis was unaffected in comparison with only cycloheximide or emetine (Fig. 1B).

Kinetics of induction of apoptosis with 2 different concentrations of puromycin. We compared the kinetics of apoptosis between 2 different concentrations of puromycin, to investigate whether a higher concentration would cause a faster induction of apoptosis in MCF-7 breast cancer cells. From 24 h and onwards there was a significant increase of the apoptosis levels for the 2 puromycin concentrations in comparison with the control value at the indicated times (Fig. 2). The largest absorbance ratio between treated and control cells was measured after 48 h for 0.5 μg/ml of puromycin (Fig. 2). Puromycin at 139 μg/ml induced apoptosis levels rather similar to those induced by 0.5 μg/ml (Fig. 2).

In a separate experiment, we measured the apoptosis induction of puromycin at 139 μg/ml after 72 and 96 h. After 72 h we registered an absorbance ratio between puromycin (n=6; Absorbance mean value=2.490; Standard deviation=0.127) and control (n=3; Absorbance mean value=1.769; Standard deviation=0.077) of 1.4. This number is evidently lower than any absorbance ratio between puromycin and control cells registered from 24 h and onwards in Figure 2. After incubation with puromycin at 139 μg/ml for 96 h, the absorbance ratio between treated (n=4; Absorbance mean value=2.029; Standard deviation=0.282) and control (n=2; Absorbance mean value=2.224; Standard deviation=0.081) cells was lowered to 0.9. However, at this time point we noticed that a vast majority of the puromycin treated cells had disrupted membranes, and thus showed signs of necrosis.

Morphological changes in MCF-7 cells after treatment with puromycin. We examined the cells after nuclear stain-

ing in order to confirm an apoptotic cell morphology. After treatment with 0.5 μg/ml of puromycin for 48 h, MCF-7 cells showed typical apoptotic features after DAPI-staining with condensed chromatin with a perinuclear arrangement (Fig. 3B). The control cells exhibited nuclei with a rather homogenously arranged chromatin (Fig. 3A). In a few cells condensed chromatine was, however, visible (Fig. 3A). If MCF-7 cells were treated with IGF-I at 10 ng/ml combined with puromycin at 0.5 μg/ml for 48 h, a minority of cell nuclei exhibited condensed chromatin (Fig. 3C). However the vast majority of the cells exhibited nuclei with rather homogenously arranged chromatin (Fig. 3C). If MCF-7 cells were treated with only IGF-I at 10 ng/ml for 48 h, all cells exhibited nuclei with homogenously arranged chromatin (Fig. 3D).

Effect of puromycin and IGF-I on induction of apoptosis. In order to investigate the apoptosis modulating effect of IGF-I in MCF-7 breast cancer cells after treatment with puromycin for 48 h, we first measured the apoptosis level at a puromycin concentration of 0.5 μg/ml without IGF-I. At this concentration we registered a high level of apoptosis (Fig. 4A and Fig. 2) and the addition of IGF-I at 10 ng/ml reduced this level to about the control value (Fig. 4A). On the other hand the addition of IGF-I at 10 ng/ml to a about 10 times higher puromycin concentration (5.7 μg/ml) did not reduce the apoptosis level although IGF-I alone significantly reduced the grade of apoptosis in comparison with the untreated control (Fig. 4B).

Effect of puromycin on the ^{3}H-leucin incorporation in the prescence or abscence of IGF-I. MCF-7 breast cancer cells were treated with puromycin for 48 h. At 2 h before the end of incubation ^{3}H-leucine was added to the cells. Puromycin at a concentration of 0.5 μg/ml reduced the protein synthesis to 34% of the control value (Fig. 4C). This effect was abolished when IGF-I (10 ng/ml) was added to the incubation medium (Fig. 4C).

Puromycin at 5.7 μg/ml and 139 μg/ml reduced the protein synthesis to 4% and 12% of the control value respectively, but the addition of IGF-I did not change the level of ^{3}H-leucine incorporation (Fig. 4D). IGF-I alone increased the protein synthesis to 196% of the control value (Fig. 4D). Thus, puromycin at 5.7 and 139 μg/ml reduced the protein synthesis to the same level as cycloheximide and emetine at the indicated concentrations (Fig. 4D and Fig. 1B).

Quantitation of IGF-I receptor m-RNA at different time points after treatment with puromycin at 0.5 μg/ml and 139 μg/ml. We wished to investigate if there were any differences in the amount of IGF-I receptor m-RNA after treatment of MCF-7 breast cancer cells with puromycin at 0.5 μg/ml and 139 μg/ml during different periods. For this purpose we measured IGF-I receptor m-RNA after hybridization between ^{35}S-UTP labelled RNA probes and total nucleic acids from the cell sample. The amount of IGF-I receptor m-RNA was determined between 1 and 72 h after incubation with puromycin at 0.5 μg/ml and 139 μg/ml (Fig. 5A). At a concentration of 0.5 μg/ml, the level of IGF-I receptor m-RNA was not significantly changed during the whole treatment time (Fig. 5A). At a concentration of 139 μg/ml, however, the amount of IGF-I receptor m-RNA was significantly reduced already after 24 h and this level was preserved during the later time points (Fig. 5A).

IGF-I receptor protein. In an attempt to correlate the reduced amount of IGF-I receptor m-RNA after puromycin treatment at 139 μg/ml to protein levels, we performed a Western blot analysis of IGF-I receptor protein after puromycin treatment at 139 μg/ml for 24 h. The experiment was repeated twice. Each sample consisted of a supernatant fraction containing loosened cells and another fraction containing cells adherent to the bottom of the Petri dishes. In the puromycin treated samples, the vast majority of cells were located in the supernatant fraction, and vice versa for the untreated control sample. Consequently, proteins in the puromycin sample originate from loosened cells in the cell growth medium and proteins in the control sample originate from cells adherent to the bottom of the Petri dishes. On two occasions, we noticed a weaker protein band in the puromycin treated sample, as shown in Fig. 5B. In another experiment the IGF-I receptor protein band was not detected in the puromycin treated sample.

Discussion

In presented work we investigated the apoptosis inducing effect of puromycin, a potent inhibitor of protein synthesis, in MCF-7 breast cancer cells, and compared its effect with cycloheximide and emetine, 2 other inhibitors of protein synthesis.

Cycloheximide at a concentration of 50 μg/ml increased the apoptosis level and the addition of IGF-I reduced this level to approximately the control value (Fig. 1A). These results are in agreement with those of other authors [14, 15]. When testing emetine at a concentration of 2 μg/ml, with or without IGF-I, the same pattern as with cycloheximide treatment could be observed (Fig. 1A). Both drugs reduced the protein synthesis to about 8% of the untreated control (Fig. 1B). Cycloheximide inhibits peptidyltransferase and emetine inhibits ribosomal translocation along the mRNA template [1, 18]. Thus, both drugs act in a rather similar way by inhibiting protein synthesis at the level of the transfer reaction.

In the case of cycloheximide and emetine, it would seem reasonable to assume that the induction of apoptosis is caused by inhibition of protein synthesis [14]. IGF-I abolished the induction of apoptosis caused by cycloheximide or

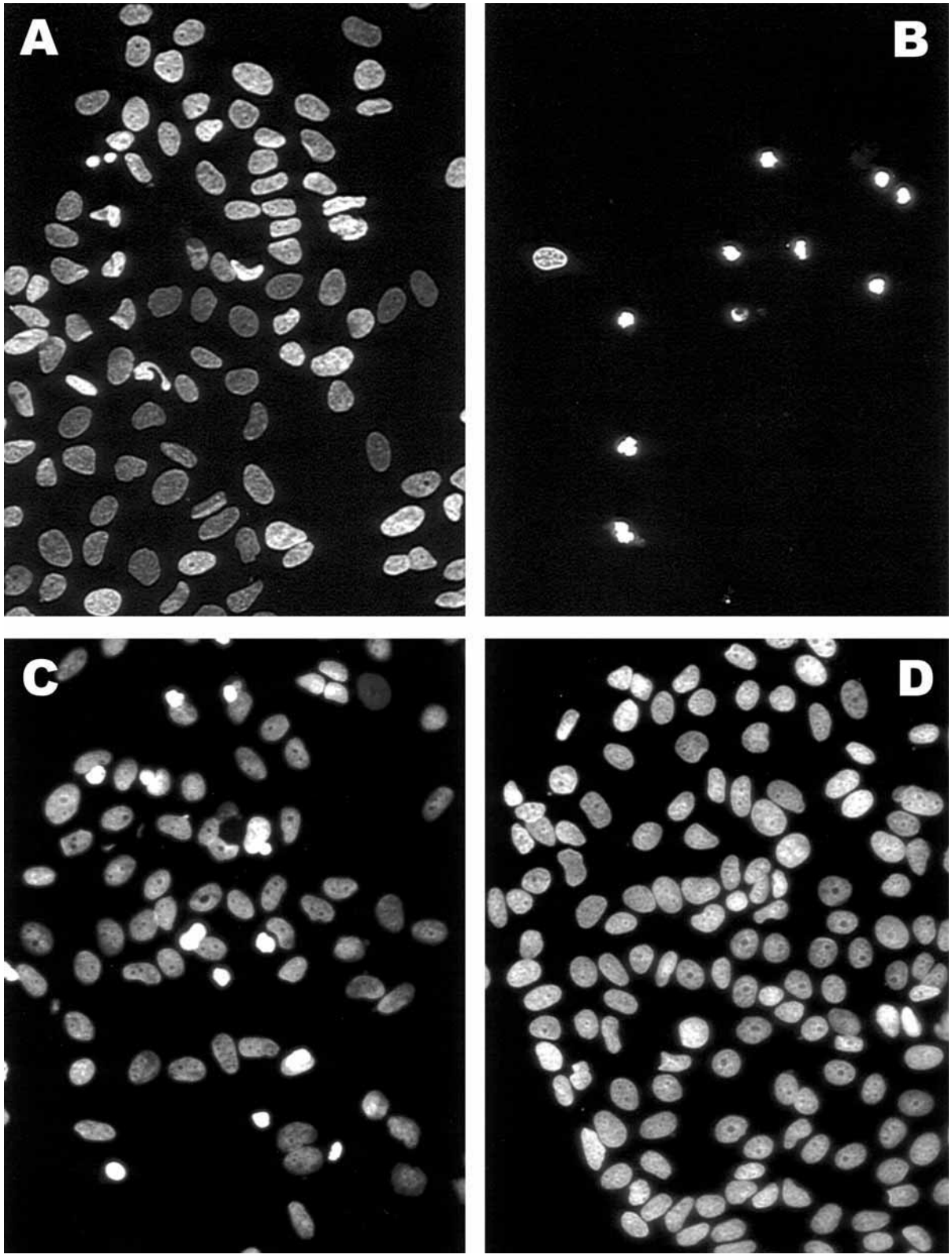

Figure 3. DAPI-staining of MCF-7 cells treated with puromycin and/or IGF-I. MCF-7 cells were handled as described in the materials and methods section. Puromycin (0.5 μg/ml) and/or IGF-I (10 ng/ml) was present during the last 48 h of incubation, whereafter the cells were DAPI-stained. Untreated control cells (A). Puromycin (0.5 μg/ml) (B). Puromycin (0.5 μg/ml) and IGF-I (10 ng/ml) (C). IGF-I (10 ng/ml) (D).

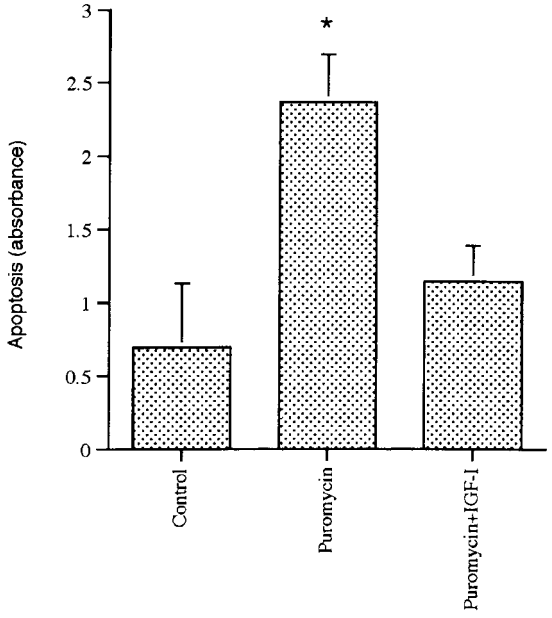

Figure 4A. Quantitation of apoptosis in MCF-7 cells after treatment with puromycin, and IGF-I. MCF-7 cells were handled as described in the materials and methods section. Puromycin (0.5 μg/ml), and IGF-I (10 ng/ml) were present during the last 48 h of incubation, whereafter apoptosis was quantified as described in the materials and methods section. Each value represents the mean +SD of triplicate wells. The experiment was repeated with similar results. *p at least <0.05 in comparison with control; Student's t-test.

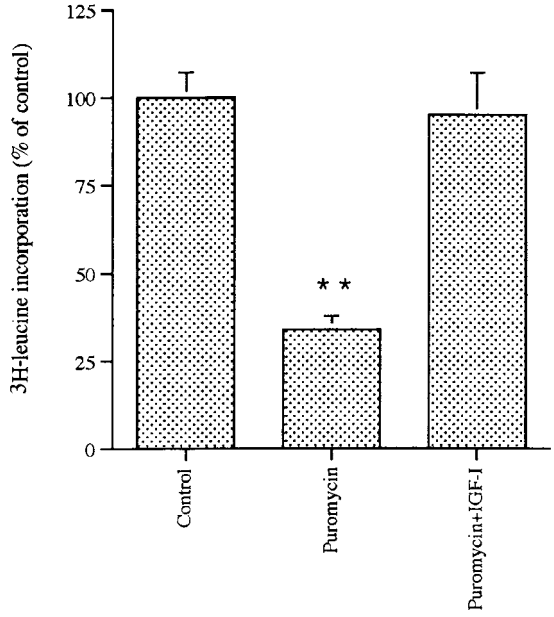

Figure 4C. Effect of puromycin, and IGF-I on ^{3}H-leucine incorporation in MCF-7 cells. The cells were handled as described in the materials and methods section. Puromycin (0.5 μg/ml), and IGF-I (10 ng/ml) were present during the last 48 h of incubation. Two hours before the end of incubation 1 μCi/ml of ^{3}H-leucine was added to each well. Each value represents the mean +SD of triplicate wells of 1 of 2 representative experiments. The mean control value corresponds to 2852 cpm. **p <0.001 in comparison with control; Student's t-test.

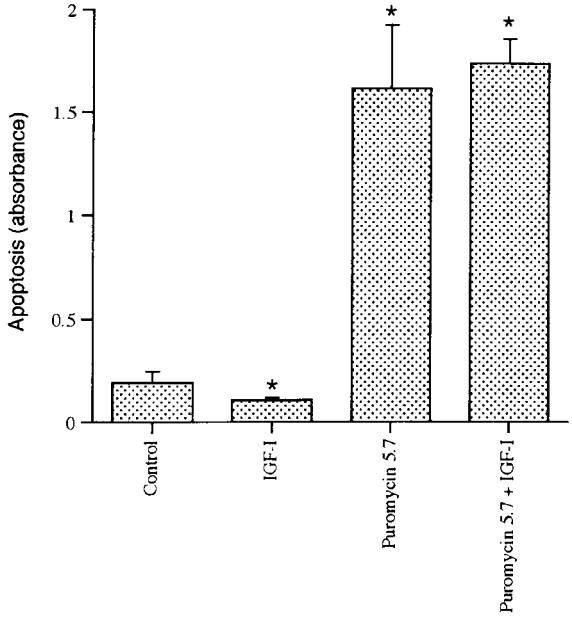

Figure 4B. Quantitation of apoptosis in MCF-7 cells after treatment with puromycin and IGF-I. MCF-7 cells were handled as described in the materials and methods section. Puromycin (5.7 μg/ml), and IGF-I (10 ng/ml) were present during the last 48 h of incubation, whereafter apoptosis was quantified as described in the materials and methods section. Each value represents the mean +SD of triplicate or quadruplicate wells of 1 of 2 representative experiments; *p at least <0.05 in comparison with control; Student's t-test.

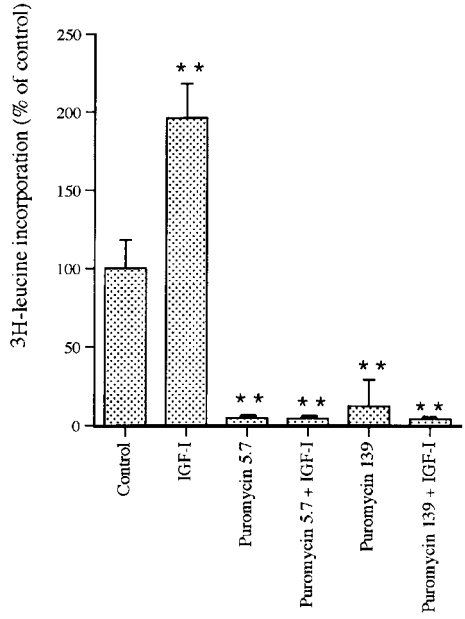

Figure 4D. Effect of puromycin, and IGF-I on ^{3}H-leucine incorporation in MCF-7 cells. The cells were handled as described in the materials and methods section. Puromycin (μg/ml), and IGF-I (10 ng/ml) were present during the last 48 h of incubation. Two hours before the end of incubation 1 μCi/ml of ^{3}H-leucine was added to each well. Each value represents the mean +SD of quadruplicate wells of 1 of 2 representative experiments. The mean control value corresponds to 1513 cpm. **p<0.001 in comparison with control; Student's t-test.

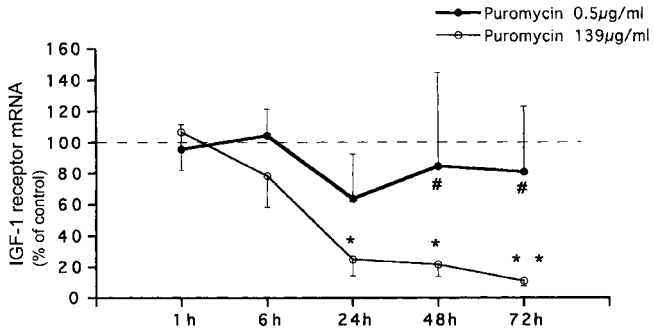

Figure 5A. Quantitation of IGF-I receptor mRNA in MCF-7 cells after puromycin treatment. MCF-7 cells were handled as described in the materials and methods section. Puromycin (μg/ml) was present at the indicated times and concentrations, whereafter the amount of IGF-I receptor mRNA was determined as described in the materials and methods section. Each value represents the mean of 3 different experiments each one performed on pooled duplicates. For each time point, a control value, also performed on pooled duplicates, was determined. Statistics were calculated using ANOVA (Fisher) ($^{*}p<0.05$, $^{**}p<0.01$ in comparison with control. #$p<0.05$ in comparison with puromycin 139 μg/ml).

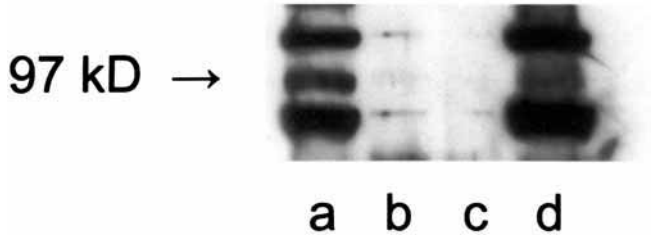

Figure 5B. Estimation of IGF-I receptor protein in MCF-7 cells after Western blotting. MCF-7 cells were treated with puromycin at 139 μg/ml for 24 h and otherwise handled as described in the materials and methods section. The band at the arrow corresponds to the IGF-I receptor protein. Control cells, adherent cell fraction (a). Control cells, supernatant fraction (b). Puromycin treated cells, adherent cell fraction (c). Puromycin treated cells, supernatant fraction (d).

emetine, although the protein synthesis was not affected (Fig. 1A and 1B). This is in agreement with earlier results for cycloheximide and MCF-7 cells, where the depressive effect on cell death, caused by insulin and IGF-I, has been assumed to be at a posttranslational level [14]. GEIER et al. suggested that IGF-I would maintain a certain protein molecule critical for cell survival [14–16]. When puromycin at 0.5 μg/ml induced a high grade of apoptosis, we did not notice a negligible amount of protein synthesised (Fig. 4A and 4C), which is why lack of some critical protein molecule does not seem plausible. When apoptosis was measured in HL-60 cells after treatment with cycloheximide or actinomycin D, the latter drug caused higher levels of apoptosis, although the inhibition of protein synthesis was significantly lower in comparison with cycloheximide [30]. Puromycin, but not cycloheximide and emetine, inhibits protein synthesis by causing premature release of truncated peptide chains [1, 18]. The truncated peptide chains have the potential of competing with the natural substrates of the ubiquitin dependent pathway including cyclins, p53, c-myc, and c-fos in the degradation [5, 9, 37] and an accumulation of these could interfere with cell cycle transitions and apoptosis [9]. DAVIDOFF and co-workers have shown that low doses of puromycin (0.5 μg/ml), in contrast to an equimolar concentration of cycloheximide (0.25 μg/ml), gave an increased accumulation of HL-60 cells in G2-phase and induction of apoptosis, although the inhibition of protein synthesis was about 50% for both puromycin and cycloheximide [9]. Furthermore, a preceding G2- arrest has been found to be linked to a subsequent induction of apoptosis in cells exposed to certain cytostatics [28, 44]. When we checked the apoptosis level, at different concentrations of cycloheximide, emetine and puromycin which gave a protein synthesis inhibition of about 50%, only puromycin showed values significantly above the control level (Data not shown). Puromycin at 139 μg/ml and 5.7 μg/ml reduced the protein synthesis to about the same level as cycloheximide and emetine at the indicated concentrations, but the addition of IGF-I neither decreased the grade of apoptosis nor increased the protein synthesis (Fig. 4B and 4D). Thus, in the case of puromycin, a reduction of the apoptosis level, caused by IGF-I, seems to parallel an increase of the protein synthesis, in contrast to cycloheximide and emetine, where the protein synthesis was not affected (Fig. 1B).

Aminopeptidases hydrolyze N-terminal amino acids of oligopeptides [8]. Puromycin-sensitive aminopeptidase is localized in the cytoplasm and the nucleus and associates with microtubules and the spindle apparatus during mitosis [8]. Puromycin competitively blocks the action of certain aminopeptidases in contrast to cycloheximide and thereby induces apoptosis at concentrations without affecting protein synthesis [8]. The apoptosis inducing capacity of puromycin might therefore only partially be caused by reduction of the protein synthesis. The inhibiting effect of aminopeptidases, and other side effects according to earlier discussion might also contribute. Cycloheximide does not affect mitochondrial protein synthesis [29] in contrast to puromycin, which at least in certain eukaryotic cells inhibits mitochondrial protein synthesis [33]. Thus, in the case of puromycin and MCF-7 cells, there might be a reduction of mitochondrial protein synthesis, which might lead to the release of mitochondrial cytochrome c and loss of mitochondrial membrane potential. Cytoplasmic cytochrome c could then act together with Apaf-1 and caspase 9 to activate caspase 3, which in the end would induce apoptotic cell death [17].

If the protein synthesis was not completly blocked by puromycin, the addition of IGF-I restored the protein synthesis and the degree of apoptosis to about the untreated control level. The addition of IGF-I could hereby increase the synthesis of the puromycin-sensitive aminopeptidases, leading to restoration of the apoptosis level. One major

signaling pathway activated by the activated IGF-I receptor is through its interaction with insulinreceptor substrate 1 or 2 leading to activation of phosphatidylinositol 3-kinase and AKT [10, 22]. Activated AKT phosphorylates and inactivates several proteins, which are involved in apoptosis [50]. A primary target is the Bcl-2 family member BAD. In its non-phosphorylated state, BAD locates at the mitochondrial membrane where it interacts with Bcl-2 and prevents Bcl-2 from performing its antiapoptotic functions [50]. Activated AKT also can prevent the initiation of the caspase cascade through phosphorylation and inactivation of caspase 9 [23]. There are some reports indicating that the activated IGF-I receptor may modulate the level of expression of the anti-apoptotic proteins BcL-XL and Bcl-2 by increasing or maintaining the protein levels after different apoptotic stimuli [36, 43]. The failure of IGF-I to affect protein synthesis and apoptosis induction at puromycin concentrations of 5.7 μg/ml and 139 μg/ml could be explained by puromycin concentrations above levels effectively inhibiting protein synthesis at a vast majority of available ribosomes. At these puromycin concentrations, when protein synthesis was completly blocked, lack of some critical protein could contribute to apoptosis induction. However this possible contribution is probably small or non-existent because the apoptosis levels at 139 μg/ml and 0.5 μg/ml (protein synthesis not completely blocked) were rather similar (Fig. 2). Furthermore, induction of apoptosis in mammalian cells causes rapid down-regulation of protein synthesis [6]. This inhibition occurs at the level of polypeptide chain initiation and is accompanied by phosphorylation and caspase-dependent cleavage of different initiation factors [6]. Thus many different circumstances can contribute to puromycin-induced inhibition of protein synthesis and puromycin-induced apoptosis. The amount of IGF-I receptor m-RNA was not affected when a puromycin concentration of 0.5 μg/ml was used (Fig. 5A), suggesting that induction of apoptosis is not via a reduced amount of IGF-I receptors [38]. However, at a puromycin concentration of 139 μg/ml there was a significant decrease of IGF-I receptor m-RNA (Fig. 5A), suggesting a reduced amount of IGF-I receptors. The Western blot analyses of the IGF-I receptor protein after puromycin treatment at 139 μg/ml for 24 h, are in support of a reduced amount of IGF-I receptors (See result 3.8 and Fig. 5B). A reduced amount of IGF-I receptors can of course not be excluded as a cause of increased apoptosis, but this does not seem plausible because the apoptosis levels for 0.5 μg/ml and 139 μg/ml of puromycin were rather similar after 48 h (Fig. 2). However a reduced amount of IGF-I receptors could contribute to the failure of IGF-I to affect protein synthesis at a puromycin concentration of 139 μg/ml (Fig. 4D). The amount of trypan blue stained cells after 48 h incubation with 139 μg/ml and 0.5 μg/ml of puromycin respectively was about the same (Data not shown), indicating that the absence of effect of IGF-I at a puromycin concentration of 139 μg/ml was not caused by an increased amount of dead cells.

Finally, as a possible future clinical application, we would like to mention protein synthesis targeting as a component of a multimodal approach in cancer treatment [4], combined with blockage of IGF-I receptor signaling. Reduction of IGF-I receptor signaling could be performed by use of a growth hormone receptor antagonist, which competes with native growth hormone for the growth hormone receptor and prevents the functional receptor dimerization [25]. This process is critical for growth hormone signal transduction and IGF-I synthesis and secretion [25].

In conclusion puromycin induces apoptosis in MCF-7 breast cancer cells at a concentration where there is still a non-negligible amount of protein synthesized in contrast to cycloheximide and emetine, where the protein synthesis is highly repressed at apoptosis inducing concentrations. IGF-I reduces the apoptosis level after puromycin treatment, but this effect occurs in parallel to increased protein synthesis, also in contrast to cycloheximide and emetine, where the protein synthesis is not affected. These differences might be explained by the specific way in which puromycin affects protein synthesis, and/or other side effects than the inhibition of protein synthesis according to earlier discussion.

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References

[1] ALBERTS B, BRAY D, LEWIS J, RAFF M, ROBERTS K et al. In: Molecular Biology of the Cell, 2nd edn., Garland Publishing, New York, 1989: 218.

[2] BARNES DM. Cells without growth factors commit suicide. Science 1988; 242: 1510–1511.

[3] BOSMAN FT, VISSER BC, VAN OEVEREN J. Apoptosis: pathophysiology of programmed cell death. Path Res Pract 1996; 192: 676–83.

[4] CARAGLIA M, BUDILLON A, VITALE G, LUPOLI G, TAGLIAFERRI P et al. Modulation of molecular mechanisms involved in protein synthesis machinery as a new tool for the control of cell proliferation. Eur J Biochem 2000; 267: 3919–3936.

[5] CIECHANOVER A, FINLEY D, VARSHAVSKSY A. Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell 1984; 37: 57–66.

[6] CLEMENS MJ, BUSHELL M, JEFFREY IW, PAIN VM, MORLEY SJ. Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells. Cell Death and differentiation 2000; 7: 603–615.

[7] COHEN JJ, DUKE RC. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol 1984, 132: 38–42.

[8] CONSTAM DB, TOBLER AR, RENSING-EHL A, KEMLER I, HERSH LB et al. Puromycin-sensitive Aminopeptidase. J Biol Chem 1995; 270: 26931–26939.

[9] DAVIDOFF AN, MENDELOW BV. Unexpected cytokinetic effects induced by puromycin include a G2- arrest, a metaphase-mitotic-arrest, and apoptosis. Leukemia Res 1992; 16(11): 1077–1085.

[10] DUDEK H, DATTA SR, FRANKE TF, BIRNBAUM MJ, YAO R et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997; 275: 661–665.

[11] DUKE RC, CHERVENAK R, COHEN JJ. Endogenous endonuclease-induced DNA fragmentation: an early event in cell mediated cytolysis. Proc Natl Acad Sci USA 1983; 80: 6361–6365.

[12] DUPONT J, KHAN J, QU B-H, METZLER P, HELMAN L, LEROITH D. Insulin and IGF-I induce different patterns of gene expression in mouse fibroblast NIH-3T3 cells: Identification by cDNA microarray analysis. Endocrinology 2001; 42(11): 4969–4975.

[13] DURNAM DM, PALMITER RD. A practical approach for quantitating specific mRNAs by solution hybridization. Anal Biochem 1983; 131: 385–393.

[14] GEIER A, HAIMSHON M, BEERY R, HEMI R, LUNENFELDT B. Insulin-like growth factor-1 inhibits cell death induced by cycloheximide in MCF-7 cells: a model system for analyzing control of cell death. In Vitro Cell Dev. Biol 1992; 28 A: 725–729.

[15] GEIER A, WEISS C, BEERY R, HAIMSOHN M, HEMI R et al. Multiple pathways are involved in protection of MCF-7 cells against death due to protein synthesis inhibition. J Cell Physiol 1995; 163: 570–576.

[16] GEIER A, HEMI R, HAIMSOHN M, BEERY R, KARASIK A. Phosphorylation of a 27-kDa protein correlates with survival of protein-synthesis-inhibited MCF-7 cells. In Vitro Cell Dev Biol-Animal 1997; 33: 129–136.

[17] GREEN D, KROEMER G. The central executioners of apoptosis: caspases or mitochondria. Trends Cell Biol 1998; 8: 267–271.

[18] GUPTA RS, SIMINOVITCH S. Mutants of CHO cells resistant to the protein synthesis inhibitors cryptopleurin and tylocrebrine: Genetic and biochemical evidence for common site of action of emetine, cryptopleurine, tylocrebrine, and tubulosine. Biochemistry 1977; 16: 3209–3214.

[19] HETTS SW. To die or not to die. An overview of apoptosis and its role in disease. JAMA 1998; 279: 300–307.

[20] IGNEY FH, KRAMMER PH. Death and Anti-Death: Tumour Resistance to Apoptosis. Nature Rev Cancer 2002; 2: 277–288.

[21] KAAKS R, LUKANOVA A. Energy balance and cancer: the role of insulin and insulin-like growth factor-I. Proc Nutr Soc 2001; 60(1): 91–106.

[22] KAUFFMANN-ZEH A, RODRIGUEZ-VICIANA P, ULRICH E, GILBERT C, COFFER P et al. Suppression of c-Myc-induced apoptosis by Ras signaling through PI(3)K and PKB. Nature 1997; 385: 544–548.

[23] KERMER P, KLOCKER N, LABES M, BAHR M. Insulin like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent akt phosphorylation and inhibition of caspase 3 in vivo. J. Neurosci 2000; 20: 722–728.

[24] KERR JFR, WINTERFORD CM, HARMON BV. Apoptosis. Its significance in cancer and cancer therapy. Cancer 1994; 73: 2013–2026.

[25] KOPCHICK JJ, PARKINSON C, STEVENS EC, TRAINER PJ. Growth hormone receptor antagonists: discovery, development, and use in patients with acromegaly. Endocr Rev 2002; 23(5): 623–646.

[26] LABARCA C, PAIGEN K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 1980; 102: 344–342.

[27] LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685.

[28] LOCK RB, ROSS WE. Inhibition of p34cdc2 kinase activity by etoposide or irradiation as a mechanism of G2-arrest in Chinese hamster ovary cells. Cancer Res 1990; 50: 3761–3766.

[29] LODISH H, BALTIMORE D, BERK A, ZIPURSKI SL, MATSUDAIRA P et al. In: Molecular cell biology, third edn., Scientific American Books, New York, 1995: 816.

[30] MARTIN SJ, LENNON SV, BONHAM AB, COHER TG. Induction of apoptosis (programmed cell death) in human leukaemic HL-60-cells by inhibition of RNA or protein synthesis. J Immunol 1990; 145: 1859–1867.

[31] MEIJERMANN I, BLOM M, DE BONT HJGM, MULDER GJ et al. Induction of apoptosis and changes in nuclear G-actin are mediated by different pathways: The effect of inhibitors of protein and RNA synthesis in isolated rat hepatocytes. Toxicol Appl Pharmacol 1999; 156: 46–55.

[32] MELTON DA, KRIEG PA, REBAGLIATI MR, MANIATIS T, ZINN K et al. Efficient in vitro synthesis of biologically active RNA, and RNA hybridization probes from plasmids containing bacteriophage SP6 promotor. Nucleic Acid Res 1984; 12: 7035–7056.

[33] NABHOLZ CE, SPEIJER D, SCHNEIDER A. Chloramphenicol-sensitive mitochondrial translation in Trypanosoma brucei. Parasitol Res 1999; 85(8-9): 779–782.

[34] NICOLETTI I, MIGLIORATI G, PAGLIACCI MC, GRIGNANI F, RICCARDI C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 1991; 139: 271–279.

[35] OTA A, ZILA S-R, ROBERTS CT JR, LEROITH D. TPA-induced neurite formation in a neuroblastoma cell line (SH-SY5Y) is associated with increased IGF-I receptor mRNA and binding. Mol Brain Res 1989; 6: 69–76.

[36] PARRIZAS M, LEROITH D. Insulin-like growth factor I inhibition of apoptosis is associated with increased expression of the bcl-xL gene product. Endocrinology 1997; 138: 1355–1358.

[37] RECHSTEINER M. Natural substrates of the ubiquitin proteolytic pathway. Cell 1991; 66: 615–618.

[38] RESNICOFF M, ABRAHAM D, YUTANAWIBOONCHAI W, ROTMAN HL, KAJSTURA J et al. The insulin-like growth factor I receptor protects tumor cells from apoptosis in vivo. Cancer Research 1995; 55: 2463–2469.

[39] RICHTER C, SCHWEIZER M, COSSARIZZA A, FRANCESCHI C. Control of apoptosis by the cellular ATP level. FEBS Lett 1996; 378: 107–110.

[40] SATOH T, ENOKIDO Y, KUBO T, YAMADA M, HATANAKA H. Oxygen toxicity induces apoptosis in neuronal cells. Cell Mol Neurobiol 1998; 18(6): 649–666.

[41] SCHAPER J, ELSASSER A, KOSTIN S. The role of cell death in heart failure. Circ Res 1999; 85: 867–869.

[42] SCHLAPBACH R, FONTANA A. Differential activity of bcl-2, and ICE enzyme family protease inhibitors on Fas and puromycin-induced apoptosis of glioma cells. Biochim Biophys Acta 1997; 1359: 174–180.

[43] SINGLETON JR, DIXIT VM, FELDMAN EL. Type 1 insulin-like growth factor receptor activation regulates apoptotic proteins. J Biol Chem 1996; 271: 31791–31794.

[44] SORENSON CM, BARRY MA, EASTMAN A. Analysis of events associated with cell cycle arrest at G2-phase and cell death induced by cisplatin. J Natn Cancer Inst 1990; 82: 749–755.

[45] TAKANO Y, HARMON BV, KERR JFR. Apoptosis induced by mild hyperthermia in human and murine tumour cell lines: a study using electron microscopy and DNA gel electrophoresis. J Pathol 1991; 163: 329–336.

[46] TAMM I, KIKUCHI T. Insulin-like growth factor-1 (IGF-I), insulin, and epidermal growth factor (EGF) are survival factors for density inhibited, quiescent Balb/c-3T3 murine fibroblasts. J Cell Physiol 1990; 143: 494–500.

[47] WANG P, VALENTIJN AJ, GILMORE AP, STREULI CH. Early events in the anoikis program occur in the absence of caspase activation. J Biol Chem 2003; 278(22): 19917–19925.

[48] WARING P. DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by raised inositol triphosphate levels. J Biol Chem 1990; 265: 14476–14480.

[49] WILLINGHAM MC. Cytochemical methods for the detection of apoptosis. J Histochem Cytochem 1999; 47: 1101–1109.

[50] VINCENT AM, FELDMAN EL. Control of cell survival by IGF signaling pathways. Growth hormone IGF Research 2002; 12(4): 193–196.

[51] WYLLIE AH. Glucocorticoid-induced thymocyte apoptosis is associated with endonuclease activation. Nature 1980; 284: 555–556.

[52] ZHOU Q, MCCRACKEN MA, STROBL JS. Control of mammary tumor cell growth in vitro by novel cell differentiation and apoptosis agents. Breast Cancer Research and Treatment 2002; 75: 107–117.