ENHANCEMENT OF LYSOSOMAL GLYCOHYDROLASE ACTIVITY IN HUMAN PRIMARY B LYMPHOCYTES DURING SPONTANEOUS APOPTOSIS

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It has been shown that lysosomes are involved in B cell apoptosis but lysosomal glycohydrolases have never been investigated during this event. In this study we determined the enzymatic activities of some lysosomal glycohydrolases in human tonsil B lymphocytes (TBL) undergoing *in vitro* spontaneous apoptosis. Fluorimetric methods were used to evaluate the activities of β-hexosaminidases, α-mannosidase, β-mannosidase, β-galactosidase, β-glucuronidase and α-fucosidase. Results show that in TBL during spontaneous apoptosis, there is a significant increase in the activity of β-hexosaminidases, α-mannosidase, β-mannosidase and β-galactosidase. Also β-glucuronidase and α-fucosidase activities increase but not in a significant manner. Further studies on β-hexosaminidases revealed that also mRNA expression of the α- and β-subunits, which constitute these enzymes, increases during spontaneous TBL apoptosis. When TBL are protected from apoptosis by the thiol molecule N-acetyl-L-cysteine (NAC), there is no longer any increase in glycohydrolase activities and mRNA expression of β-hexosaminidase α- and β-subunits. This study demonstrates for the first time that the activities and expression of some lysosomal glycohydrolases are enhanced in TBL during spontaneous apoptosis and that these increases are prevented when TBL apoptosis is inhibited.

Apoptosis fulfils fundamental functions in the B cell compartment, contributing to the maintenance of self-tolerance, ensuring the homeostatic control of B cell populations and guaranteeing the specificity and affinity maturation of the antibody response (1-3). Therefore, given the importance of apoptosis in B cell physiology and pathology, knowledge about new molecules and/or enzymatic activities involved in B cell apoptosis could help to define targets for future therapeutic approaches in cancer, transplantation and autoimmune diseases.

For many years it has been thought that mitochondrial dysfunctions and caspase activation are the central events in the B cell apoptosis (4-6), which can be induced by several stimuli such as B cell antigen receptor engagement (4-5, 7-8), Fas/CD95 ligation (9-10), or in the absence of the appropriate survival factors (11-12).

Key words: lysosomal glycohydrolases, B lymphocytes, apoptosis

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Recently, a role of lysosome and lysosomal enzymes has been proposed in B cell apoptosis, both in immature and germinal center B cells. One report demonstrated that germinal center B cell apoptosis requires both cathepsin and caspase activity, with cathepsins acting downstream of caspase-3 and directly connected with endonuclease activity (13). In immature B cells, lysosomal changes and release of cathepsin B into the cytosol are involved both in the initiation of apoptosis, representing a primary apoptotic step without any alteration in mitochondria (14), and the post-mitochondrial execution of apoptosis where instead of caspases, cathepsin B is activated (15).

In addition to B lymphocytes, the role of lysosome and lysosomal enzymes in apoptosis, has become clear in several other cell types, so that the existence of a lysosomal apoptosis pathway is now generally accepted (16). Important mediators of this pathway are the cathepsins, proteases which after a partial selective permeabilization of the lysosomal membrane are released into the cytosol where, by different mechanisms, they contribute to apoptosis (17-19), and some lipid hydrolases, including acidic sphingomyelinase and ceramidase which, leading to generation of the bioactive sphingolipids ceramide and sphingosine, mediate pro-apoptotic effects of various stimuli (20-22). On the contrary, little information is available on the relationship between lysosomal glycohydrolases and the apoptotic process.

These enzymes in physiological conditions play a fundamental role in the degradation of the oligosaccharide structures of glycoproteins, glycolipids and proteoglycans, the basis of the normal cellular constituent turnover and the cellular homeostasis of glycosilation (23).

Given the involvement of lysosomes in B cell apoptosis and the fact that lysosomal glycohydrolases have never been explored in B cell apoptosis, we questioned whether changes in these activities could be associated with this event. To this end, in the present study we examined the activities of lysosomal glycohydrolases: β-hexosaminidases (Hex, EC 3.2.1.52), α-mannosidase (α-man, EC 3.2.1.24), β-mannosidase (β-man, EC 3.2.1.25), β-galactosidase (β-gal, EC 3.2.1.23), α-fucosidase (α-fuc EC 3.2.1.51) and β-glucuronidase (β-gluc, EC 3.2.1.31), using as a model human tonsil B lymphocytes (TBL) undergoing spontaneous apoptosis *in vitro*.

Results show that in TBL during spontaneous apoptosis, activities of Hex, α-man, β-man and β-gal increase significantly compared to freshly isolated cells. Also α-fuc and β-gluc activities increase but not in a significant manner. A more detailed analysis of Hex indicated that their enhanced enzymatic activity could be due to a gene up-regulation. When we examined a condition in which TBL are protected from apoptosis by the thiol molecule N-acetyl-L-cysteine (NAC) (24), we found that the increases in glycohydrolase activity and gene expression were inhibited.

MATERIALS AND METHODS

Materials

4-Methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside (4-MU-GlcNAc-6-SO_4) was from Toronto Research Chemicals Inc. 4-Methylumbelliferyl-β-N-acetylglucosaminide (4-MU-GlcNAc), 4-methylumbelliferyl-α-D-mannoside (4-MU-α-mann), 4-methylumbelliferyl-β-D-mannoside (4-MU-β-mann), 4-methylumbelliferyl-β-D-galactoside (4-MU-β-gal), 4-methylumbelliferyl-α-N-fucoside (4MU-α-fuc), 4-methylumbelliferyl-β-N-glucuronide (4-MU-β-gluc), 4-methylumbelliferone, Nonidet P-40, protease inhibitor cocktail for mammalian cell extracts, N-acetyl-L-cysteine (NAC), propidium iodide (PI), L-glutamine and bovine serum albumin (BSA) were from Sigma Chemicals Co. (St. Louis, Mo). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin were from Biochrom KG Seromed-Bio (Milan, Italy). Bio-Rad protein assay reagent was from Bio-Rad Laboratories (Richmond, CA). RNeasy Mini Kit and Taq PCR Core Kit were from Qiagen (Hilden, Germany). SuperScript™ II Reverse Transcriptase RNase H⁻ was from Invitrogen Life Technologies, (Carlsbad, CA). All reagents were of analytical grade.

Cell isolation and culture

Mononuclear cells from surgically removed tonsils were isolated by gentle mincing followed by centrifugation on Ficoll-Hypaque density gradients (Amersham Pharmacia, Uppsala, Sweden). Cell suspension was T cell-depleted by two cycles of rosetting with neuraminidase-treated sheep erythrocytes and monocyte-depleted by plastic adherence. This B cell enriched population was further fractionated on a discontinuous gradient of 60, 50 and

30% Percoll (Pharmacia). Cells recovered between the 60 and 50% layers, considered high density cells, were used in this study. These B cells were shown to be >98% pure on flow cytometry by CD19 staining, with <1% CD14+ and <2% CD3+. Cells were cultured at 37°C in a humid 5% CO_2 atmosphere at a concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium). NAC was soluble in culture medium, and the pH was adjusted to 7.4 before use. NAC was added at the beginning of culture and maintained throughout the test. Cell viability was evaluated by the trypan blue dye exclusion assay.

Preparation of cell extracts for enzymatic assay

Cell samples were centrifuged at 1600 g for 7 min at 4°C. The cell pellets were washed twice with ice-cold Dulbecco's phosphate-buffered saline (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.5, PBS) and resuspended in lysis buffer (sodium phosphate 10 mM, pH 6.0, 0.1% (v/v) NP-40, protease inhibitor cocktail) at a concentration of 10^8 cells/ml. After incubation for 1 hour on ice, cell lysates were subjected to three sonications, 15 sec each, then centrifuged at 12000 g for 20 min at 4°C. The supernatants (cell extracts) were recovered and used for protein determination and enzymatic assays.

Glycohydrolase assays

Total Hex, Hex A, α-man, β-man, β-gal, α-fuc and β-gluc activity levels were measured using 3 mM solutions of the fluorogenic substrates 4-MU-GlcNAc (total Hex), 4-MU-GlcNAc-6-SO_4 (Hex A), 4-MU-α-mann, 4-MU-β-mann, 4-MU-β-gal, 4-MU-α-fuc and 4-MU-β-gluc, respectively. In all assays, at the end of the reaction period, 2.85 ml of 0.2 M glycine-NaOH buffer, pH 10.4, was added. Fluorescence of the liberated 4-methylumbelliferone was measured on a Perkin Elmer LS B50 fluorimeter (excitation, 360 nm; emission, 446 nm). One unit (U) is the amount of enzyme that hydrolyses 1 µmol of substrate/min at 37°C. Protein content was determined by the Bradford method using BSA as standard. Specific activity was expressed as mU/mg of protein.

Assessment of apoptosis

Apoptosis was assessed by flow cytometric quantification of nuclei with hypodiploid DNA content, as described by Nicoletti et al. (25). Briefly, 3×10^5 cells were harvested, permeabilized in PI staining solution (PI 50 µg/ml, sodium citrate 0.1%, Triton X-100 0.1%) and stored at 4°C in the dark overnight. The PI fluorescence of individual nuclei was measured by flow cytometry with standard FACScan equipment (Becton Dickinson, San Jose, CA).

RNA extraction and RT-PCR

RNA was extracted from 10^8cells with RNeasy Mini Kit. cDNA was obtained by reverse transcription of RNA with random hexamer primers and SuperScript™ II RNaseH- according to the manufacturer's procedure. Finally, PCR was performed with Taq PCR Core Kit, using the following primers: for Hex α-subunit , forward primer 5'-ggcgtacaataaattgaacgtgtt-3' and reverse primer 5'-cataaagtcctggatctctgggtt-3'; for Hex β-subunit, forward primer 5'-agtcctgccagaatttgatacc-3' and reverse primer 5'-ctattccacgttcgaccatcc-3'; for β-actin, forward primer 5'-tgacggggtcacccacactgtgcccatcta-3' and reverse primer 5'-ctagaagcatttgcggtggacgatggaggg-3'. Densitometric evaluation of bands was obtained with Kodak Digital Science 1D (Eastman Kodak, Rochester, NY).

Statistical analysis

Experiments to assess glycohydrolase activity and hypodiploid DNA content were repeated in eight different TBL isolates. Results are shown as the mean ± S.E. of eight experiments. Statistical differences were evaluated using paired Student's *t* test. RT-PCR experiments were performed in five different TBL isolates. Data reported are those of a typical experiment.

RESULTS

Levels of glycohydrolase activity during spontaneous TBL apoptosis

Specific activities of the lysosomal glycohydrolases total Hex, Hex A, α-man, β-man, β-gal, α-fuc and β-gluc were determined in freshly isolated TBL and TBL cultured *in vitro* for 24 and 48 hours, using 4-methylumbellyferyl derivatives of the appropriate monosaccharides. Since Hex are present in normal cells as two major isoenzymes, Hex A and Hex B, consisting of αβ and ββ dimers respectively, and displaying different substrate specificity, two substrates were employed for their analysis: 4-MU-GlcNAc, which being hydrolysed by both the α- and β- subunits, measures total Hex activity, and its sulphated derivative 4-MU-GlcNAc-6-SO_4, which reacting only with the α-subunit measures activity of Hex A (26-28). Results in Fig. 1A show that the total Hex and Hex A activities increased progressively in cultured TBL compared to freshly isolated cells ($P<0.05$ at 24 and $P<0.01$ at 48 hours). Moreover, the analysis carried out with

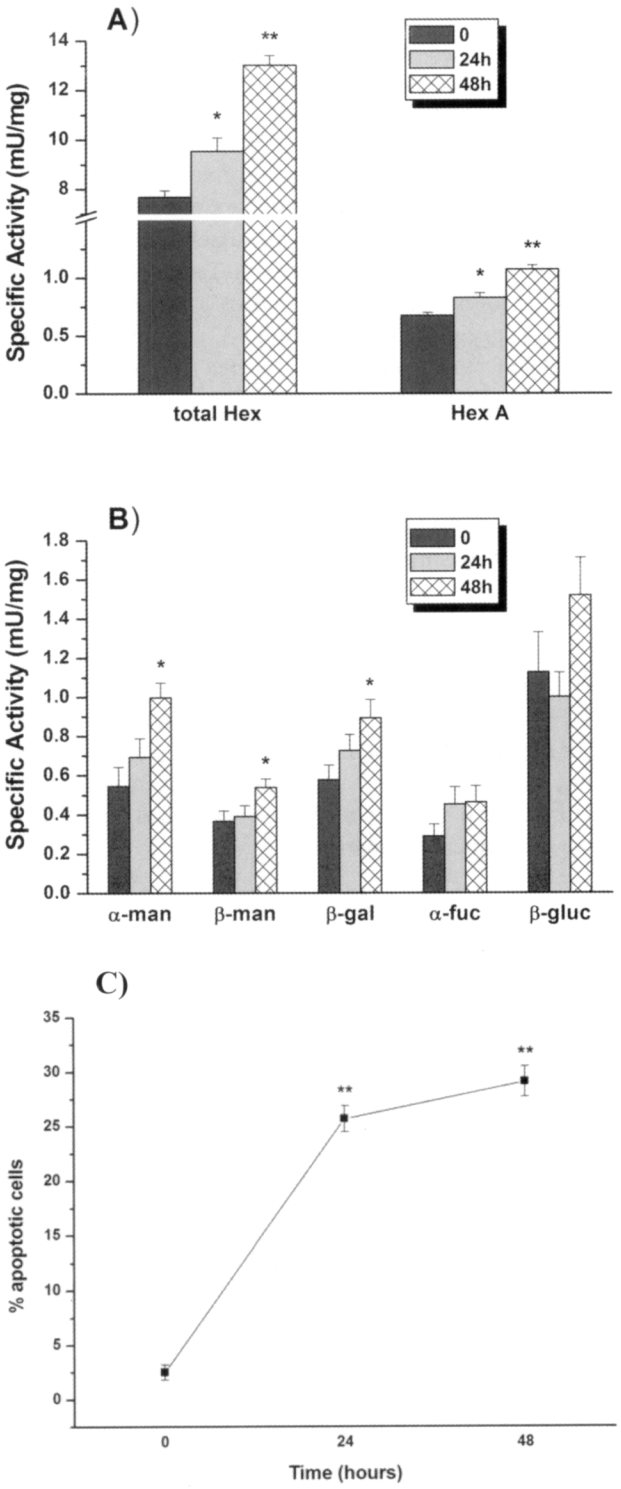

Fig. 1. Lysosomal glycohydrolase activities in TBL undergoing spontaneous apoptosis. *Glycohydrolase activities (A, B) and apoptosis (C) were determined in freshly isolated TBL and TBL cultured in complete medium for the indicated times. (A, B) Activities of the indicated enzymes were measured in cell extracts using fluorogenic substrates as described in Materials and Methods, and were expressed as mU/mg of protein (specific activity). (C) Apoptosis was evaluated measuring the percentage of hypodiploid nuclei by flow cytometry. (A, B, C) The results are shown as the means ± S.E. of eight independent experiments. Each experiment used TBL from a different subject. *, P < 0.05 and **, P < 0.01 (cultured TBL vs freshly isolated TBL) according to Student's t test.*

the two substrates indicates that activities of both Hex subunits are involved in this increase already at 24 hours (Fig. 1A). The α-man, β-man and β-gal activities also increased with respect to freshly isolated TBL, reaching significant values at 48 hours (P<0.05) (Fig. 1B). α-Fuc and β-gluc activities increased during TBL spontaneous apoptosis but not in a significant manner (Fig. 1B). Flow cytometric analysis of hypodiploid DNA content, performed in the same cultures where enzymatic activity was detected, shows that the percentage of apoptotic TBL increased strongly in the 24- and 48-hours cultures with respect to freshly isolated cells (P< 0.01 at 24 and 48 hours) (Fig. 1C).

NAC prevents the enhancement of lysosomal glycohydrolase activity

We demonstrated recently that TBL apoptosis is inhibited by NAC, which exerts its anti-apoptotic activity in a concentration-dependent manner (Fig. 2) (24). The 20 mM NAC induced the strongest reduction of hypodiploid DNA content in TBL compared to controls (P<0.01 at 24 and 48 hours) (Fig. 2). The 5 mM NAC still exerted a significant anti-apoptotic effect but only at 48 hours (P<0.01), while the 1 mM NAC had no effect on TBL apoptosis (Fig. 2). Because NAC inhibits TBL apoptosis by inhibiting crucial apoptotic molecular events (24), in the present study we question whether the enhancement of glycohydrolase activities observed in TBL undergoing apoptosis can also be affected by NAC. To verify this, activities of total Hex, Hex A, α-man, β-man and β-gal were assessed in TBL cultured for 24 and 48 hours in the presence of the above-indicated NAC concentrations, and compared with cells cultured in complete medium. Results in Fig. 3 show that the increases in total Hex and Hex A activities occurring during spontaneous apoptosis

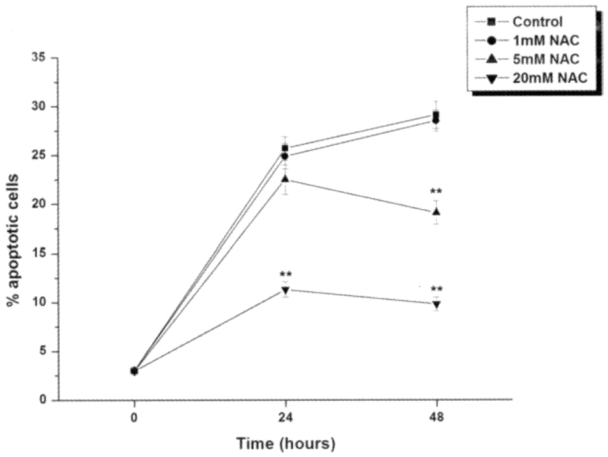

Fig. 2. NAC inhibits spontaneous apoptosis in TBL. *Apoptosis was evaluated in freshly isolated TBL and TBL cultured for different times with or without the indicated concentrations of NAC, measuring the percentage of hypodiploid nuclei by flow cytometry. The results are shown as the means ± S.E. of eight independent experiments. Each experiment used TBL from a different subject. **, P < 0.01 (NAC-treated TBL vs control TBL at each time-point) according to Student's t test. The effect of NAC 1 mM was not significant.*

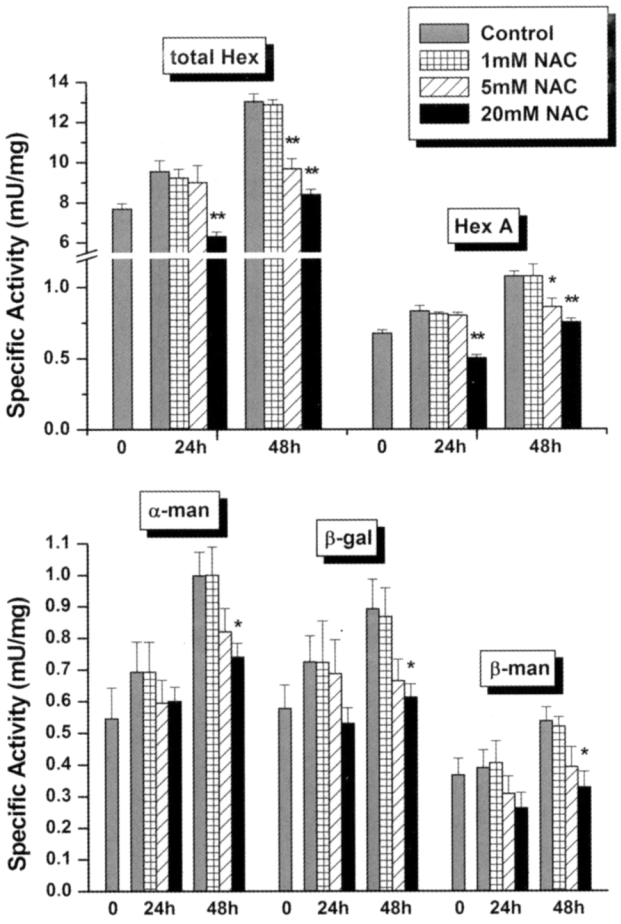

Fig. 3. NAC inhibits the enhancement of lysosomal glycohydrolase activity. *Glycohydrolase activity was evaluated in freshly isolated TBL and TBL cultured for different times with or without the indicated concentrations of NAC. Enzymatic activities were measured in cell extracts using fluorogenic substrates as described in Materials and Methods, and were expressed as mU/mg of protein (specific activity). The results are shown as the means ± S.E. of eight independent experiments. Each experiment used TBL from a different subject. *, P < 0.05 and **, P < 0.01 (NAC-treated TBL vs control TBL at each time-point) according to Student's t test. The effect of NAC 1 mM was not significant.*

were significantly inhibited by NAC 20 mM, both at 24 and 48 hours (P<0.01). Also NAC 5 mM at 48 hours significantly reduced the increases in total Hex (P<0.01) and Hex A (P<0.05) activities, while NAC 1 mM had no effect on these glycohydrolases at any time examined (Fig. 3). Also the increases in the specific activities of α-man, β-man and β-gal were significantly inhibited by NAC, but only at 48 hours (P< 0.05) and at a 20 mM concentration (Fig. 3). Data in Fig. 2 and Fig. 3 suggest that NAC inhibits both TBL apoptosis and the increases in Hex activities in a similar concentration-dependent manner and with similar kinetics.

Hex α- and β-subunit transcripts in TBL during spontaneous apoptosis and effect of NAC

To determine whether the increase in Hex activity observed during TBL spontaneous apoptosis and its inhibition by NAC were correlated with altered transcript levels, we performed RT-PCR analysis of both Hex α- and β-subunit transcripts in freshly isolated TBL and TBL cultured for 24 and 48 hours with or without NAC 20 mM. We used primers which allowed amplification of a 0.435 kb cDNA fragment of the HEXA gene product and a 0.771 kb cDNA fragment of the HEXB gene product. The β-actin cDNA fragment amplified as an internal control was 0.660 kb. Results in Fig. 4A, B show a progressive increase of both Hex α- and β-subunit transcripts in cultured TBL, compared to freshly isolated cells. Moreover, in TBL cultured with NAC, expression of both Hex α- and β-subunit transcripts resulted

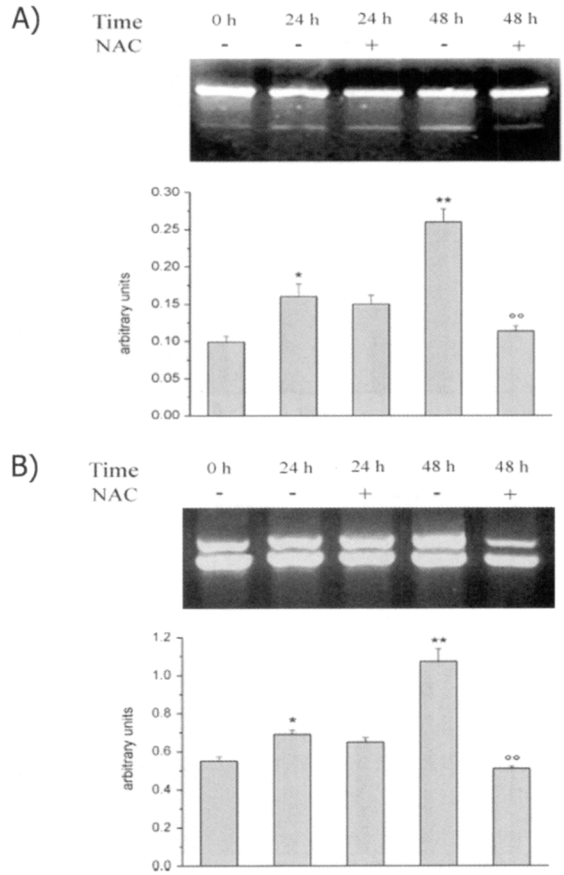

Fig. 4. Hex α- and β-subunit mRNA expression in TBL during spontaneous apoptosis and effect of NAC. *Total RNA was extracted from freshly isolated TBL and TBL cultured with or without NAC 20 mM for 24 and 48 hours. For RT-PCR analysis 0.5 μg of total RNA were employed. Ten percent of the RT reaction mixture was amplified by PCR and analysed on 1.5% agarose gel. As control, β-actin expression was determined for each sample in the same reaction. (A) Electrophoretic analysis of Hex α-subunit and β-actin mRNA amplification products. The reaction yielded the bands of the expected molecular weight (0.435 kb for Hex α-subunit, 0.660 kb for β-actin). (B) Electrophoretic analysis of Hex β-subunit and β-actin mRNA amplification products. The reaction yielded the bands of the expected molecular weight (0.771 kb for Hex β-subunit, 0.660 kb for β-actin). (A,B) One representative experiment of five with similar results is shown. Densitometric analysis was performed for each blot and the density of each band, represented by the ratio between the fluorescence intensity of Hex subunit and β-actin mRNA amplification products, is given as the mean ± S.E. of five experiments. Lane designations are identical for blots and histograms. *, $P < 0.05$ and **, $P < 0.01$ (cultured TBL vs freshly isolated TBL); °°, $P < 0.01$ (NAC-treated vs untreated TBL at 48 hours) according to Student's t test.*

reduced at 48 hours to levels similar to those of freshly isolated cells (Fig. 4A, B). Quantification of the transcripts by densitometric analysis is reported in the histograms (Fig. 4A, B).

DISCUSSION

Increasing evidence suggests that lysosomal functions play a role in the initiation and execution of the apoptotic program. In particular, changes in lysosomal membrane permeabilization and lysosomal pH, and activation of some cathepsins, such as cathepsin D, an aspartyl protease, and cathepsins B and L, two cysteine proteases, are involved in the apoptosis of several cell types, including B lymphocytes (13-19). However, a correlation between lysosomal glycohydrolase activities and apoptosis has never been demonstrated in these cells.

Based on these observations, the present study was undertaken to determine whether there were any alterations in the activities of some lysosomal glycohydrolases in TBL during *in vitro* spontaneous apoptosis. Results provide the first evidence that in TBL undergoing spontaneous apoptosis, levels of total Hex, Hex A, α-man, β-man and β-gal activities were increased with respect to freshly isolated TBL. Also β-gluc and α-fuc activities increased but not in a significant manner. We then focused our attention on Hex enzymes since they showed the highest activity among the glycohydrolases examined in TBL. Furthermore, activities of total Hex and Hex A increased progressively with TBL apoptosis, and these increases were significant already at 24 hours.

In normal human cells and tissues, Hex are present as two major isoenzymes, Hex A and Hex B, accounting for about 60-70% and 30-40% of the total Hex activity, respectively. They are composed of two subunits, α and β, that are encoded by two closely related genes (29-30). Although each subunit contains an active site, their dimerization is required for the acquisition of Hex A (αβ) and Hex B (ββ) enzymatic activity. When properly folded and assembled, the enzymes are transferred

to the Golgi apparatus for the synthesis of mannose 6-phosphorylated residues, which are recognized by the mannose 6-phosphate receptors which target the enzymes to lysosomes, where they undergo a final processing to their mature forms (31). In physiological conditions, both Hex isoenzymes cleave terminal β-glycosidically-linked N-acetyl-glucosamine and N-acetyl-galactosamine residues from a number of glycoconjugates, including oligosaccharides, glycoproteins, glycolipids, and glycosaminoglycans, so playing an important role in the normal turnover of basic cellular components (32). Instead, only Hex A, together with a specific activator protein (33), can degrade the natural substrate GM2 ganglioside, one of the plasma membrane glycosphingolipids, molecules that play a fundamental role in cell-cell interactions and inter- and intra-cellular signalling (34- 35). In addition, glycosphingolipid catabolism gives rise to ceramide, an important transducer and modulator of apoptotic cell death (36).

Recently, a mature lysosomal Hex A, active against GM2 ganglioside, was found associated with the plasma membrane of normal human cells, suggesting its possible involvement in regulating cell-cell and/or cell-microenvironment interactions (37), which are known to be crucial for cell survival, in both physiological and pathological conditions (38-39).

All the above observations and our results, indicating the progressive increase of Hex activity during TBL apoptosis, prompted us to perform further studies at transcriptional level. Evaluation of mRNA expression of Hex α- and β-subunits performed by RT-PCR, shows that both Hex subunit transcripts increase in the TBL undergoing spontaneous apoptosis compared to fresh cells and suggests that the increased enzymatic activity may be a consequence of a transcriptional up-regulation.

Our findings that lysosomal glycohydrolase expression and activities increase in TBL during spontaneous apoptosis, pose the question of whether these enzymes may be involved in the apoptotic process of these cells. Recently, it was proposed that some lysosomal mannose 6-phosphorylated enzymes play a role in the apoptotic cascade induced by TNFα in fibroblasts (40). This was suggested by the fact that some apoptotic events triggered by TNFα in normal fibroblasts, including processing of caspase-8 and the pro-apoptotic protein BID, and the release of cytochrome c from mitochondria into the cytosol, were strongly reduced in fibroblasts isolated from patients affected with mucolipidosis II, with a deficient activity of almost all lysosomal hydrolases (40). Furthermore, it seems that the site of action of mannose 6-phosphorylated enzymes in the apoptotic cascade triggered by TNFα is possibly upstream of mitochondria (40).

In TBL, further studies are necessary to understand whether lysosomal enzymes contribute to spontaneous apoptosis and, if so, how they may be connected with some fundamental events occurring during this process and consisting of a caspase-8-independent activation of the pro-apoptotic protein BID, mitochondrial dysfunctions, as indicated by the cytochrome c release from mitochondria into the cytosol, and the activation of caspase-3 and -7, as reported in our recent study (24).

Recently, we demonstrated that TBL apoptosis is prevented by the thiol molecule NAC, which exerts its anti-apoptotic action independent of its antioxidant properties and inhibits the above indicated pro-apoptotic events, and also induces changes in several regulatory components of the apoptotic process (24).

When in the present study, we analysed the effect of NAC on total Hex, Hex A, α-man, β-man and β-gal activities, using the concentration which induces the highest anti-apoptotic effect, we found that the increases of all these activities were also inhibited by NAC and lowered to values similar to those of freshly isolated TBL. Furthermore, NAC prevents the increase in Hex activity in a concentration-dependent manner similar to when it inhibits TBL apoptosis and with similar kinetics. However, we do not know whether the inhibition of glycohydrolase activity by NAC is associated with its anti-apoptotic activity, and further investigation will be undertaken to establish this. Conversely, it is evident that the inhibition of Hex activity by NAC may be due, at least in part, to an inhibited gene expression, as suggested by the fact that NAC also significantly reduced the increased Hex α- and β-subunit mRNA expression at 48 hours. These results suggest the ability of NAC to interfere with some molecular events involved in HEXA and HEXB gene transcription and are in agreement with a

growing body of evidence supporting the ability of thiol molecules to interact with several components and/or steps of the intracellular signalling pathways (41-43).

In conclusion, TBL spontaneous apoptosis is accompanied by an up-regulated expression and activity of some lysosomal glycohydrolases. Furthermore, these events are prevented by NAC which protects TBL from apoptosis. Whether the enhancement of lysosomal glycohydrolase activities has a specific role in TBL apoptosis could be a matter of investigation.

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