Cathepsin B expression and down-regulation by gene silencing and antisense DNA in human chondrocytes

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Cathepsin B, a marker of the dedifferentiated chondrocyte phenotype, contributes to cartilage destruction in osteoarthritis and pathological proteolysis in rheumatoid arthritis and cancer. In search of possible means for neutralizing the action of this enzyme, we compared its expression, biosynthesis and distribution in articular chondrocytes and two lines of immortalized human chondrocytes. Native articular chondrocytes in primary culture and the polyclonal T/C-28a2 chondrocyte cell line were similar with respect to the number of endosomes and lysosomes, the distribution of three alternatively spliced cathepsin B mRNA forms, and the cathepsin B activity. In contrast, the clonal C-28/I2 cell line contained four times higher levels of intracellular cathepsin B activity, slightly higher numbers of endosomes and lysosomes, and uniform distribution of all three cathepsin B transcripts and thus resembled subcultured chondrocytes at an early stage of dedifferentiation. Transfection of T/C-28a2 chondrocytes with double-stranded cathepsin B mRNA resulted in inhibition of cathepsin B biosynthesis by up to 70 % due to RNA interference, and single-stranded antisense DNAs of various sizes decreased cathepsin B biosynthesis by up to 78 %. An antisense oligonucleotide designed to hybridize to the end of cathepsin B's exons 1 and the beginning of exon 3 was successful in specifically inhibiting the mRNA splice variant lacking exon 2. These results indicate that cathepsin B expression and activity may be targeted for gene silencing by RNA interference and antisense DNA in chondrocytes. Furthermore, the differential expression and distribution of cathepsin B and presence of the necessary molecular apparatus for gene silencing in the immortalized human chondrocyte cell lines indicate that they may serve as a useful model for studying the function of relevant enzymes in cartilage pathologies.

Key words: cartilage, cysteine peptidase, osteoarthritis, proteolysis, RNA interference.

INTRODUCTION

In early studies, the cysteine peptidase cathepsin B was recognized as a mediator of pathological proteolysis in articular cartilage [1,2]. Significantly higher levels of this enzyme have been found in human osteoarthritic cartilage compared with normal tissue [3–6], as well as in experimental osteoarthritis [7]. Chondrocytes cultivated in monolayers progressively display an altered phenotype characterized by down-regulation of cartilage-specific type II collagen, by the expression of non-specific type I and III collagens [8], and by the development of a stiff cytoskeleton in parallel with an increased number of intracellular organelles of the endosomal-lysosomal lineage [9]. In the dedifferentiated chondrocyte phenotype, cathepsin B production is noticeably enhanced [10,11] by a cytokine-independent mechanism [12], which directly depends on overexpression of cathepsin B mRNA [13] and protein synthesis [14]. The distribution of three mRNA forms, alternatively spliced in the 5′-untranslated region (5′-UTR), is also altered as the chondrocytes lose the differentiated phenotype [15]. Some studies have shown that changes in the properties of articular chondrocyte phenotype are associated with events typically observed in osteoarthritis, such as the promotion of angiogenesis and cartilage mineralization [16], and the maintenance of chronic matrix degradation in spite of active remodelling [11].

RNA interference (RNAi) is a recently discovered method that allows specific gene silencing through targeted degradation of mRNA by cognate double-stranded RNA (dsRNA) [17,18]. RNAi has proven very useful for gene inactivation in invertebrates and plants, with less success so far in vertebrates. Antisense DNA and oligonucleotides are a convenient approach for the selective down-regulation of protein expression. In the case of cathepsin B, antisense DNA was utilized to demonstrate the involvement of the enzyme in tumour cell invasion [19–21] and tumour cell apoptosis [22].

Immortalized chondrocytes are a powerful tool for studying normal and pathological processes in cartilage in a reproducible manner [23–26], and avoid many problems associated with the use of primary chondrocyte cultures. In this study we have characterized cathepsin B expression and intracellular distribution in two immortalized human chondrocyte lines compared with primary articular chondrocytes and show the applicability of antisense and RNAi methods for down-regulating enzyme biosynthesis.

EXPERIMENTAL

Cell culture and cathepsin B assay

Human articular chondrocytes were prepared from pooled knee and hip cartilage of normal donors and cultured as described previously [9]. The immortalized human chondrocyte cell lines T/C-28a2 and C-28/I2 were established from primary cultures of juvenile costal chondrocytes by transfection with vectors encoding the simian virus 40 large T antigen [23]. Cells were cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (1:1, v/v) with 10 % fetal calf serum (FCS) at 37 °C in a humidified incubator with a 5 %

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; eIF-2, eukaryotic initiation factor-2; FCS, fetal calf serum; RNAi, RNA interference; RT, reverse transcriptase; siRNA, small interfering RNA; ssDNA, single-stranded DNA; 5′-UTR, 5′-untranslated region.

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CO_2/95 % air atmosphere at seeding densities of 2×10^4 cells/cm² and 2.4×10^4 cells/cm² for T/C-28a2 and C-28/I2, respectively, in 25 or 75 cm² culture flasks. Maintenance of the cell line was achieved by weekly passaging; 5 days after seeding two-thirds of the medium was withdrawn and replaced with fresh medium, and on the seventh day the cells were trypsinized. For analysis of cathepsin B activity in the intracellular pool and in culture medium, the T/C-28a2 cells were seeded at 1.2×10^5 cells/well, C-28/I2-cells at 1.5×10^5 cells/well and articular chondrocytes at 1×10^5 cells/well in 12-well plates in 1.0 ml of DMEM/F-12 supplemented with 10 % FCS. After 2 days in culture, the cells were washed twice with serum-free medium and incubated with 1.0 ml/well of serum-free medium for 24 h. Cathepsin B activity was measured in supernatants and cell layers treated as described in [9], and the units of cathepsin B activity were expressed as pkat/10^6 cells.

Plasmids, primers and oligonucleotides

The human cathepsin B mRNA sequence, to which all sequences shown in this study refer, is the sequence of 2002 nucleotides under accession number M14221 at the EMBL/GenBank/DDBJ Nucleotide Sequence Databases. Total mRNA was isolated from a sample of freshly excised normal human liver with the Oligotex direct mRNA mini kit from Qiagen (Basel, Switzerland). cDNA was prepared by reverse transcription using the oligo(dT) primer (5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAA-GCTTTTTTTTTTTTTTTTT-3′) for rapid amplification of cDNA ends. A first PCR was then performed with the 3′ primer (5′-CCAGTGAGCAGAGTGACG-3′) and a 5′ primer corresponding to positions 1–19 of cathepsin B exon 1 (5′-AATTC-CGCGGCAACCGCTC-3′). A second PCR was performed with the 3′ primer (5′-GAGGACTCGAGCTCAAGC-3′) and a 5′ primer corresponding to positions 24–47 of cathepsin B exon 1 (5′-AACGCCAACCGCTCCGCTGCGCGC-3′). The obtained cDNA (ps-hCBM14/24-2002 in Figure 1A), encoding human preprocathepsin B, was cloned in both orientations into the multiple cloning site of the expression plasmid pSPORT 1 (Gibco-BRL, Basel, Switzerland), and in the pGEM-T plasmid (Promega, Wallisellen, Switzerland), and sequences were confirmed by DNA sequencing (Microsynth, Balgach, Switzerland). A cDNA encoding human preprocathepsin B missing exon 2 was prepared in the same way [ps-hCBM14/22-2002(−2) in Figure 1B]. A control sense oligonucleotide (5′-CTGCAGCGCTGG-GTGGATCGAGGA-3′) was synthesized, and consisted of 12 nucleotides at the end of cathepsin B exon 1 (positions 70–81 of M14221) and of the first 12 nucleotides of cathepsin B exon 3 (positions 170–181 of M14221). The corresponding antisense oligonucleotide was 5′-TCCTAGATCCACCCAGCGCTGCA-G-3′. Primers and oligonucleotides were synthesized by Microsynth, and the oligonucleotides were purified by HPLC and PAGE.

Preparation of dsRNA

The cDNA template obtained from the plasmid pSPORT 1 by cleavage at the *Sph*I site was used for mRNA transcription using the T7 RNA polymerase and other reagents of Promega. *In vitro* synthesis of the capped mRNA transcript was performed using the m⁷G cap analogue and T7 RNA polymerase (Promega; protocol no. 9PIP207, method C, available online at www.promega.com). The product was purified with the Quick Spin columns of Boehringer Mannheim (Mannheim, Germany) followed by 2-fold precipitation with ethanol. The same procedure was applied to synthesize sense and antisense cathepsin B mRNA. dsRNA was prepared by boiling for 5 min at 100 °C

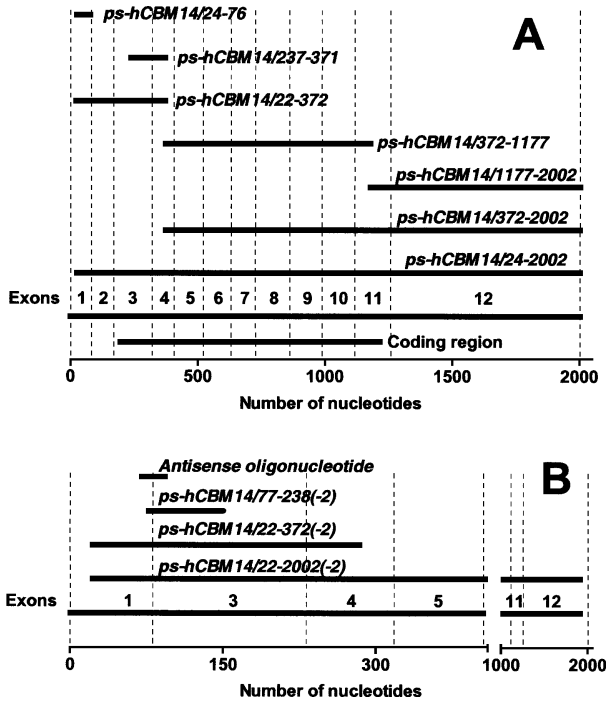

Figure 1 Clones of human cathepsin B used in this study

The clone names are composed of the name of the vector into which they were introduced (ps = pSport), human cathepsin B (hCB), the cathepsin B sequence with accession number M14221 (M14; EMBL/GenBank/DDBJ), followed by a slash and the first and last nucleotide numbers in the M14221 sequence. (**A**) Clones derived by deletions from the full-length cDNA. (**B**) Clones bridging exons 1 and 3, and missing exon 2. The antisense nucleotide bridging exons 1 and 3, with 12 nucleotides in each of the two exons, is also shown.

equimolar concentrations of sense and antisense mRNA, and allowing the mixture to slowly regain room temperature. Unpaired mRNA portions were removed by digestion with RNase H (Roche, Basel, Switzerland).

Preparation of single-stranded DNA (ssDNA)

The cDNA clones for human cathepsin B with and without exon 2 introduced into the pGEM-T plasmid were cut into various fragments with the appropriate restriction enzymes, ligated again into the pGEM-T plasmid, and their composition determined by sequencing. The clones obtained in this study are summarized in Figure 1. After cleaving at the end of the encoding sequence with the appropriate restriction enzyme, ssDNA was amplified by PCR using either primer M13*for* to obtain sense ssDNAs or M13*rev* to obtain antisense sequences. After purification on agarose gels and extraction of the ssDNAs with an agarose gel extraction kit (Boehringer Mannheim), the ssDNA concentration was measured with a Gene Quant spectrometer (Amersham Biosciences, Dübendorf, Switzerland).

Transfection of immortalized chondrocytes for ELISA detection

The T/C-28a2 chondrocytes were seeded at 8×10^4 cells/35 mm culture dish, and maintained for 48 h in 3.0 ml of DMEM/F-12 containing 10 % FCS at 37 °C in a humidified incubator. The medium was replaced 1 h before the transfection procedure and then substituted with the transfection mixtures prepared as follows: 0.4 μg of ssDNA in 100 μl of EC buffer, 3.2 μl of enhancer and 12.5 μl of Effectene (all from the Effectene kit,

Qiagen; basic method according to the manufacturer's specifications); 4 μg of dsRNA in 100 μl of EC buffer, 8 μl of enhancer and 12.5 μl of Effectene; sense or antisense oligonucleotide at a final concentration of 5 μM in 100 μl of EC buffer, 8 μl of enhancer and 12.5 μl of Effectene. To each of these mixtures, 0.6 ml of culture medium was added. Transfection was initiated by adding to each well 1.6 ml of fresh medium, followed by slow drop-wise addition of the ssDNA or dsRNA transfection reagent. After 4 h of incubation the cells were washed twice with PBS, and 1 ml of 50 mM sodium acetate buffer, containing 2 mM EDTA, pH 6.0, was added to each well. After sealing the culture plates, freezing at -80 °C and thawing, the plates were sonicated in a water bath for 20 min. The remaining adherent cells were scraped off and the suspension was sonicated for 30 s with a needle sonicator operating at 50 W, frozen at -80 °C, thawed and sonicated again before storage at -80 °C. Transfection efficiency was tested with the green fluorescent protein method by incubating the chondrocytes, as described above, with 0.4 μg of DNA/pEGFP-N3 vector (Clontech, Basel, Switzerland) for various times.

Monitoring the activation of the interferon response by dsRNA

The possibility that the interferon cascade was activated by dsRNA was examined by measuring the phosphorylation of the α subunit of the general translation initiation factor eIF-2 (eukaryotic initiation factor-2) as one of the first elements of the cascade. We sought phosphorylated eIF-2 by Western blotting of cell extracts. 8×10^4 T/C-28a2 chondrocytes were seeded in 35 mm culture dishes, transfected as described above with 1 and 2 μg of a dsRNA specific for the coding region of firefly luciferase, with the corresponding ssRNA, or just treated with the transfection reagents not containing RNA, and incubated for 24 h. The coding sequence of luciferase from the pGL3-control vector (Promega) was cloned into the pGEM vector (Promega) by PCR. Sense and antisense ssRNAs were then synthesized *in vitro* with T7 and Sp6 polymerases (Promega), and converted into dsRNA as described above. Cell lysates were electrophoresed on 12 % acrylamide SDS/PAGE gels under reducing conditions. The primary antibodies were immunoaffinity-purified rabbit anti-phospho-eIF-2α (Ser-51) IgG (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Bands were visualized by the light-emitting, non-radioactive method (BM chemiluminescence POD; Boehringer) using anti-rabbit peroxidase secondary antibodies, and images captured on Agfa Curix Ortho HTA films with an intensifying screen. Cell lysates were also analysed by cathepsin B ELISA as described in the preceding section. A positive control for eIF-2 phosphorylation was run by transfecting the human A431 keratinocyte cell line, originally derived from a squamous carcinoma of the skin, with 100 μg/ml dsRNA in the form of polyinosinic-polycytidilic acid (Sigma, Buchs, Switzerland) for 4 h.

Detection of cathepsin B synthesis after transfection of immortalized chondrocytes

The T/C-28a2 chondrocytes were seeded at 8×10^4 cells/dish in 35 mm culture dishes and maintained for 48 h as described above. The medium was replaced 1 h before the transfection procedure with medium containing 4 % FCS but without methionine and cysteine. The cells were transfected as described above using medium without methionine and cysteine, in the presence of 10 % FCS. FCS was first dialysed against PBS. After transfection, 100 μCi of Promix (Gibco) containing [^{35}S]methionine and [^{35}S]cysteine was added to each well. Following 4 h of incubation, the cells were washed twice with PBS and lysed with lysis buffer (10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 0.2 % SDS and 1 % Triton X-100 [27]) and homogenized with a 21 gauge $\times$ 3.3 cm needle. Protein G–Sepharose (10 μl; Amersham Biosciences) was then added to the homogenates and the suspension incubated with shaking for 20 min to eliminate non-specific binding to Sepharose. Specific immunoprecipitation of the enzyme was performed with 15 μl of affinity-purified sheep anti-rabbit cathepsin B antibodies cross-reacting with the human enzyme [28] and 15 μl of Protein G–Sepharose, for 1 h at room temperature under gentle shaking. The resulting precipitate was collected by centrifugation at 600 ***g***, washed twice with PBS and then resuspended in 20 μl of SDS reducing sample buffer, and boiled for 5 min at 100 °C. After centrifugation for 5 min at 14000 ***g*** the supernatant was applied to SDS/PAGE (10 % gel). After electrophoresis, the gels were rapidly stained with Coomassie Blue and dried for 2 h at 80 °C. The gels were exposed on a PhosphorImager screen for 1–5 days and analysed with Molecular Dynamics ImageQuant version 4.2a software (Amersham Biosciences).

Other methods

Vital staining of cultured cells with Acridine Orange and staining for intracellular cathepsin B activity by a fluorogenic method were performed as described previously [9]. The primers and the detailed method for semi-quantitative mRNA analysis by reverse transcriptase (RT)-PCR using the 18 S rRNA as an internal standard, as well as the method for *in situ* RT-PCR, were described elsewhere [15]. Quantitative evaluation of cathepsin B protein in cell extracts was carried out using a commercial ELISA kit (KRKA, Novo Mesto, Slovenia) after diluting the samples to 0.02 mg of protein/ml. mRNA secondary structures were calculated using the program RNAstructure, version 3.6 [29]. Cathepsin B levels measured in chondrocyte extracts were normalized to the DNA content of the cells, which was determined fluorimetrically by the PicoGreen dsDNA method (Molecular Probes, Leiden, The Netherlands). A calibration curve between cell count and dsDNA content was constructed and used for normalization.

RESULTS

Cathepsin B activities were measured in primary and subcultured normal human articular chondrocytes and various subcultures of T/C-28a2 and C-28/I2 immortalized chondrocytes to compare the levels of enzyme expression over time. A characteristic of both immortalized chondrocyte cell lines was the low level of secreted cathepsin B, roughly corresponding to that of primary articular chondrocytes. Contrary to primary cultures of articular

Table 1 Cathepsin B activity in human chondrocytes

Data for the immortalized chondrocyte cell lines T/C-28a2 and C-28/I2 represent average activities in three subcultures, spanning six different passages. Normal articular chondrocytes (AC) were examined from the primary to the second subculture. Means $\pm$ S.D. are shown.

	Cathepsin B activity (pkat/10^6 cells)	
Chondrocyte type	Intracellular	Secreted
T/C-28a2	4.5 $\pm$ 2.2	2.8 $\pm$ 0.6
C-28/I2	18.6 $\pm$ 3.7	4.0 $\pm$ 0.7
AC, primary	5.2 $\pm$ 1.3	3.5 $\pm$ 0.2
AC, first subculture	25.0 $\pm$ 1.7	13.7 $\pm$ 1.1
AC, second subculture	50.4 $\pm$ 1.1	23.7 $\pm$ 3.0

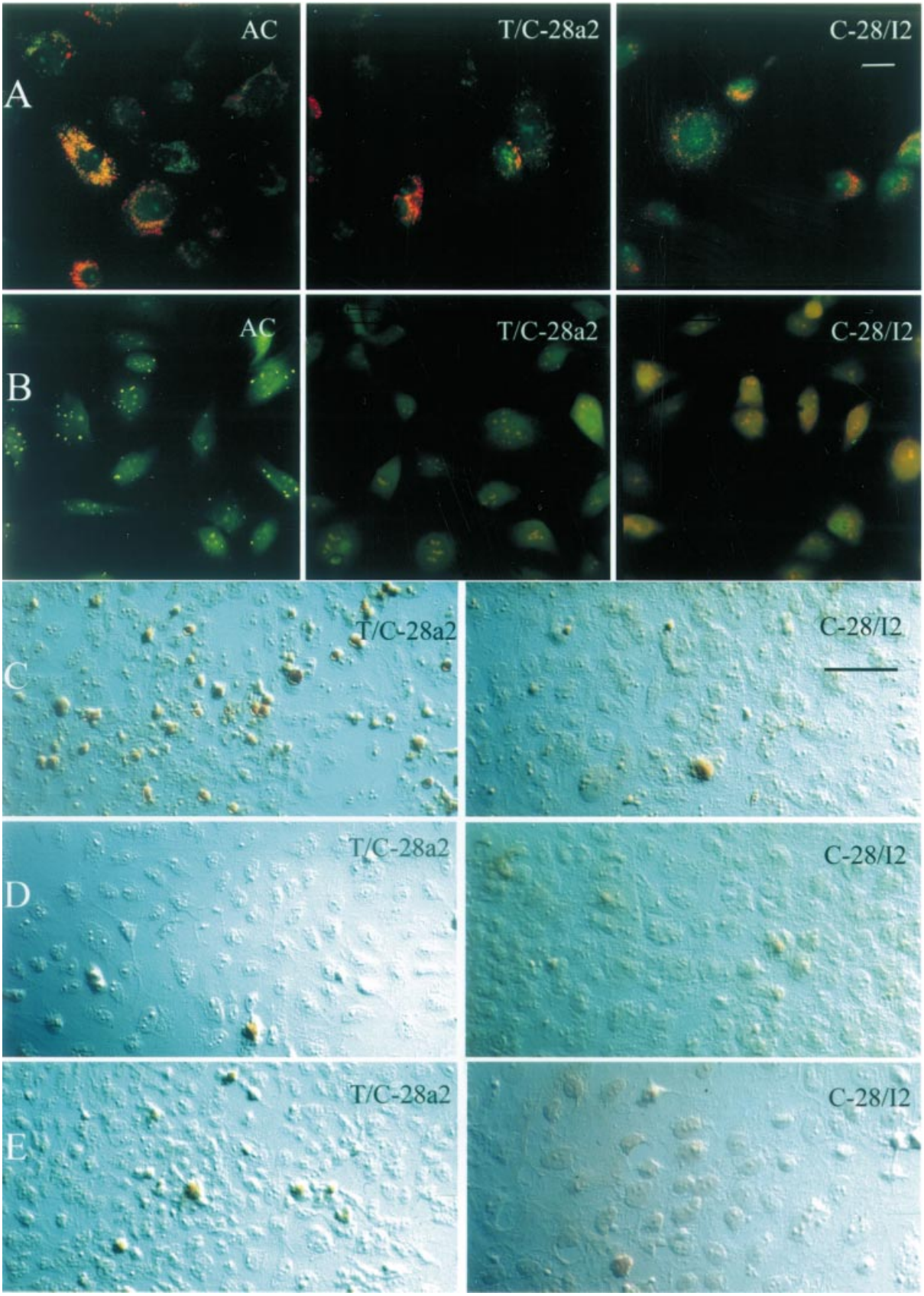

Figure 2 For legend, see facing page

Table 2 Abundance of three cathepsin B mRNA splice variants in human chondrocytes

The transcripts CB, CB(−2) and CB(−2,3) represent the full transcript, and transcripts lacking exon 2, or exons 2 and 3, respectively. Values represent percentages of the total cathepsin B mRNA (±S.D.) for T/C-28a2 and C-28/I2 immortalized chondrocytes in comparison with cultured articular chondrocytes (AC).

	Total cathepsin B mRNA (%)		
Chondrocyte type	CB	CB(−2)	CB(−2,3)
T/C-28a2	54.9±2.8	5.6±1.1	39.5±3.9
C-28/I2	53.5±11.1	1.6±2.3	44.9±13.4
AC, primary*	53	9	38
AC, second subculture*	27	31	42

* From [15].

chondrocytes, which progressively increased cathepsin B production going from a primary to a second subculture, the immortalized chondrocytes maintained constant levels of expression of cathepsin B from one generation to another (Table 1). However, the intracellular pool of cathepsin B activity in C-28/I2 cells was about four times higher than that in T/C-28a2 cells or primary articular chondrocytes. Cathepsin B is secreted by chondrocytes as the inactive proenzyme, but this undergoes rapid autoactivation under the conditions of the assay in the presence of a synthetic substrate [15]. Thus, the secreted enzyme shown in Table 1 represents active cathepsin B.

The acidic compartment, comprising endosomes and lysosomes, the final storage location for cathepsin B and other lysosomal enzymes, was visualized by vital staining with Acridine Orange. T/C-28a2 and C-28/I2 chondrocytes contained discrete amounts of organelles with uniform cytoplasmic distribution (Figure 2A). On average, C-28/I2 cells contained slightly more endosomes/lysosomes than T/C-28a2 cells. In comparison, dedifferentiated articular chondrocytes exhibited a higher number of Acridine Orange-positive granules. Cytochemical staining *in situ* with a fluorogenic substrate [10] was used to visualize intracellular cathepsin B activity in chondrocytes. Neither immortalized chondrocyte cell line exhibited the typical cathepsin B activity pattern observed in dedifferentiated chondrocytes (Figure 2B). T/C-28a2 cells were completely negative for intracellular cathepsin B activity, and no more than 1% of C-28/I2 chondrocytes contained just one needle-like fluorescent deposit.

The proportions of the cathepsin B mRNA species alternatively spliced in the 5′-UTR, i.e. the full-length transcript, CB, the variant lacking exon 2, CB(−2), or the variant lacking exons 2 and 3, CB(−2,3), were measured by semi-quantitative RT-PCR. The predominant splice variants in both T/C-28a2 and C-28/I2 cells were CB and CB(−2,3), while the species lacking exon 2 was less represented, recalling the properties of articular chondrocytes in primary culture (Table 2). The presence of the three cathepsin B mRNA splice variants in T/C-28a2 and C-28/I2 chondrocytes was confirmed by *in situ* RT-PCR, a method which only provides qualitative results but shows clearly which cells are actually responsible for the expression of a particular mRNA. In

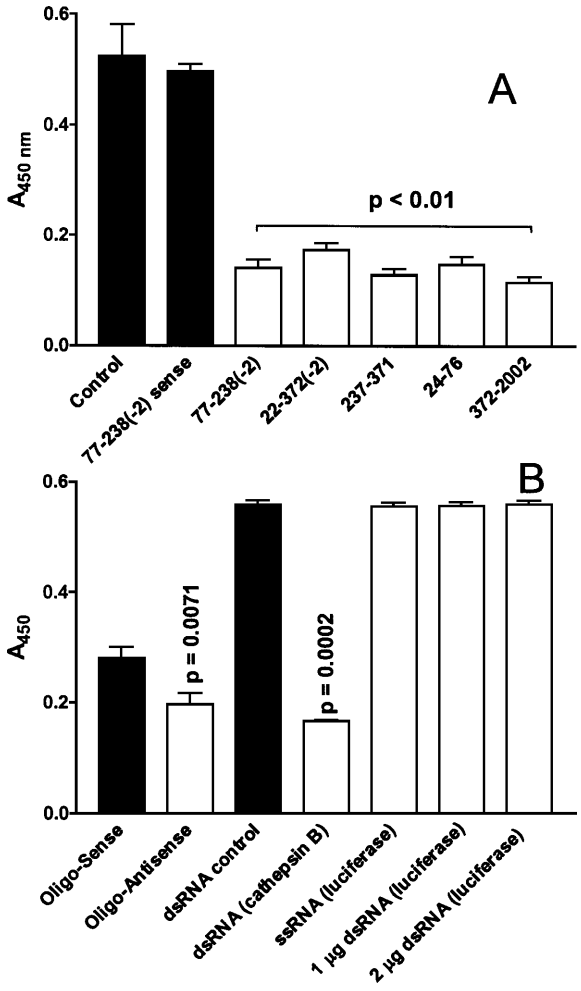

Figure 3 Inhibition of cathepsin B gene expression by antisense ssDNA and dsRNA in human immortalized T/C-28a2 chondrocytes

Mean values ±S.D. of cathepsin B levels determined by ELISA in three separate experiments. The result with sense ssDNA is shown just for one clone. The common prefix ps-hCBM14/ was omitted from the clone names for practical purposes. (**A**) Cells transfected with ssDNA. (**B**) Cells treated with a sense and the corresponding antisense oligonucleotide bridging 12 bases each in exons 1 and 3; cells treated with dsRNA corresponding to a nearly full-length cathepsin B cDNA, as well as with the ssRNA and the dsRNA for firefly luciferase.

both cell lines, numerous stained cells contained the full transcript CB and the CB(−2,3) variant, while staining for the CB(−2) variant was less prominent (Figures 2C–2E). The essential difference between T/C-28a2 and C-28/I2 cells was the proportion of stained versus non-stained cells. In T/C-28a2 chondrocytes only a subpopulation of the cells was stained, with more cells positive for CB and CB(−2,3) than for CB(−2). On the contrary, in the C-28/I2 line most of the cells were stained positively for the three transcripts, albeit with considerable differences between the three variants, and with a fairly small intensity. This fact may not be well appreciated in the right-hand

Figure 2 Acidic compartment, intracellular cathepsin B activity and *in situ* RT-PCR of cathepsin B mRNA splice variants in T/C-28a2 and C-28/I2 chondrocytes

(**A**) Vital staining of endosomes and lysosomes with Acridine Orange. (**B**) Staining for intracellular cathepsin B activity (AC, articular chondrocytes in a second subculture). (**C**–**E**) Localization of cathepsin B mRNA splice variants by *in situ* RT-PCR. (**C**) CB, full-length transcript; (**D**) CB(−2), transcript lacking exon 2; (**E**) CB(−2,3), transcript lacking exons 2 and 3. Scale bars: (**A**, **B**) the bar in the top right panel of (**A**) corresponds to 25 μm; (**C**–**E**) that in (**C**) corresponds to 30 μm.

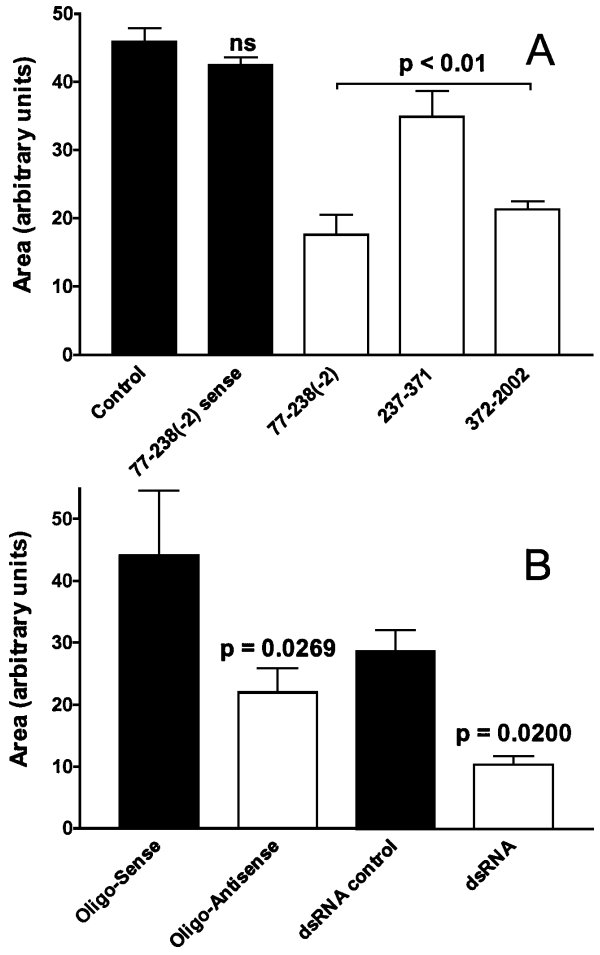

Figure 4 Inhibition of cathepsin B gene expression by antisense ssDNA and dsRNA in human immortalized T/C-28a2 chondrocytes

Means ± S.D. of cathepsin B levels determined by radioactive labelling with [^{35}S]Met and [^{35}S]Cys in three separate experiments. The result with sense ssDNA is shown just for one clone. The common prefix ps-hCBM14/ was omitted from the name of the clones for practical purposes. (**A**) Cells transfected with ssDNA. (**B**) Cells treated with a sense and the corresponding antisense oligonucleotide bridging 12 bases each in exons 1 and 3; cells treated with dsRNA corresponding to a nearly full-length cathepsin B cDNA.

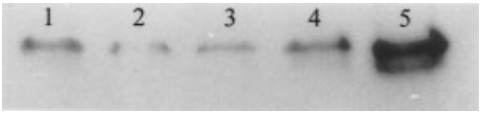

Figure 5 Western blots for the analysis of eIF-2 phosphorylation

Cell extracts of T/C-28a2 chondrocytes treated with RNA corresponding to the coding region of firefly luciferase and relative controls (lanes 1–4): lane 1, ssRNA; lane 2, dsRNA (2 μg/well); lane 3, (1 μg/well); lane 4, transfection medium without RNA. Lane 5, cell extract of human A431 cells treated with 100 μg/ml dsRNA in the form of polyinosinic/polycytidilic acid. The primary antibodies were rabbit anti-phospho-eIF-2α (Ser-51) IgG, and bands were visualized by the BM chemiluminescence POD method with anti-rabbit peroxidase secondary antibodies. The extract of an equal number of cells was applied to each lane.

panels of Figures 2(C)–2(E) because of the weak staining, which was a pale brown in most of the cells. The negative controls, in which the primers were omitted, demonstrated that the reactions carried out were specific, whereas the positive controls omitting DNase, in which both RNA and DNA were revealed, were always very intensely stained (results not shown).

Antisense methods were effective in reducing cathepsin B biosynthesis (Figures 3 and 4). Preliminary experiments were performed by Western blotting of cell extracts following 24 h of incubation after transfection, and demonstrated that all ssDNA species shown in Figure 1 could down-regulate cathepsin B expression to various degrees (results not shown). For instance, clones ps-hCBM14/22-372, ps-hCBM14/372-1177 and ps-hCBM14/1177-2002, which were not used for the other experiments described below, inhibited cathepsin B expression by 33 %, 49 % and 45 %, respectively. For quantitative evaluation, ELISA measurements and experiments with radioactively labelled amino acids were used, as shown in Figures 3(A) and 4(A) respectively. The rationale for using two detection methods was that, with ELISA, both the enzyme already present in the cells before the beginning of the treatment and the enzyme synthesized during the treatment were measured. Conversely, radiolabelling revealed only the enzyme produced after the beginning of the treatment. The antisense ssDNAs containing or not exon 2 tested in the ELISA experiments inhibited cathepsin B expression by 67–78 % (Figure 3A), whereas less inhibition was measured by the radioactive method with three ssDNAs (Figure 4A). The variable effects observed for the same treatment using radiolabelling and ELISA as detection methods were plausibly due to method-intrinsic reasons, but followed a common trend. The cathepsin B levels measured after transfection with sense probes were all statistically indistinguishable from the levels measured in untreated cells. Only the results using the sense clone ps-hCBM14/77-238(−2) are shown (Figures 3A and 4A).

Sense and antisense strands of cathepsin B mRNA were prepared using a cDNA spanning exons 1–12, i.e. clone ps-hCBM14/24-2002 in Figure 1. After promoting double-strand formation, the dsRNA was transfected into the T/C-28a2 chondrocytes. Cathepsin B expression was monitored by ELISA and radioactive labelling with [^{35}S]Met and [^{35}S]Cys followed by immunoprecipitation and PAGE (Figures 3B and 4B). RNAi down-regulated cathepsin B with respect to controls, with inhibitory effects between 64 and 70 % depending on the detection method. Variations of the transfection protocol and treatments for up to 24 h did not affect viability, morphology, adherence and DNA content of the cells. Cytoplasmic dsRNA, as a common component of virus-infected cells, can induce a sequence of signalling events, which begin with activation of RNA-dependent protein kinase ('PKR') and 2′, 5′-oligonucleotide synthetase, and end up with the induction of type I interferon [30,31]. In order to ascertain whether the inhibition of cathepsin B biosynthesis observed after transfection of chondrocytes with dsRNA was specific or was rather interferon-dependent, we examined the effects of a dsRNA not related to cathepsin B. We used a dsRNA corresponding to the coding region of firefly luciferase and analysed the cathepsin B content of cell lysates by ELISA after an incubation of 24 h following transfection. As shown in Figure 3(B), transfection of immortalized chondrocytes with the ssRNA or the dsRNA for luciferase had no effect on cathepsin B biosynthesis.

We also verified the possible activation of interferon response by measuring the phosphorylation of the α subunit of the general translation initiation factor eIF-2 as one of the first effects of protein kinase PKR after transfecting chondrocytes with luciferase dsRNA (Figure 5). A positive control for eIF-2 phosphorylation, consisting of human A431 cells transfected with polyinosinic/polycytidilic acid dsRNA, is also shown in

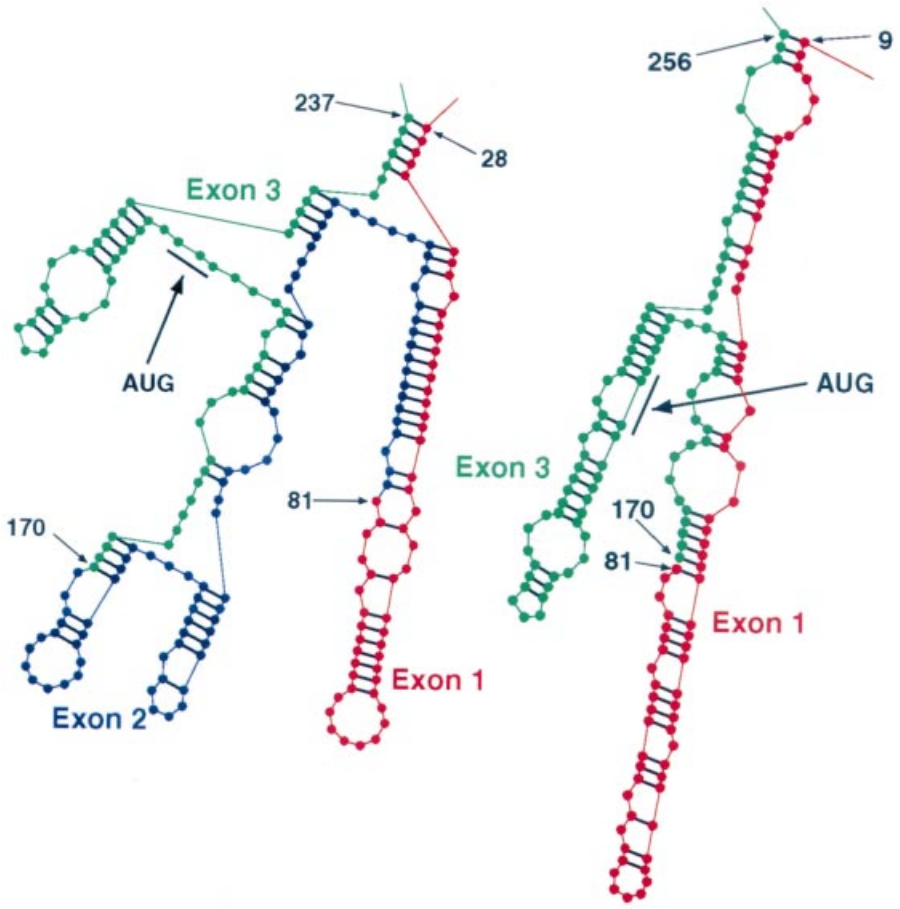

Figure 6 Predicted secondary structures of human cathepsin B mRNA splice variants CB and CB(−2)

The structures were calculated with the program RNAstructure, version 3.6 [29]. Only the region between exons 1 and 3 is shown here. Left-hand panel, full transcript; right-hand panel, transcript lacking exon 2. Nucleotide numbering is according to cathepsin B sequence under accession number M14221 (EMBL/GenBank/DDBJ). AUG, transcription initiation codon.

Figure 5. On the basis of the experiments shown in Figures 3(B) and 5 we conclude that transfection of immortalized chondrocytes with dsRNA, at the concentrations and with incubation times specified in the Experimental section, resulted in specific effects related to cathepsin B gene silencing, not to non-specific effects or to activation of the interferon type I cascade.

The antisense oligonucleotide 5′-TCCTAGATCCACCCAG-CGCTGCAG-3′ was specific for hybridizing to the cathepsin B mRNA splice variant CB(−2) because it formed a bridge between 12 nucleotides in exon 1 and 12 in exon 3. Secondary structures for human cathepsin B mRNA splice variants CB and CB(−2) were calculated by the dynamic programming algorithm of Mathews et al. [29], which uses a free-energy-minimization method. The structure of the two species was identical in the region between exons 4 and 12, but differed markedly in the 5′-UTR depending on the presence or not of exon 2. Figure 6 shows that the designed antisense oligonucleotide can hybridize to the CB(−2) variant, whereas hybridization with the full-length variant is less favourable. We recall that specificity of antisense oligonucleotides is only guaranteed when at least 15–19 consecutive nucleotides are complementary to RNA, and that 12 nucleotides possibly binding to either exon 1 or exon 3 of the full-length transcript (left-hand structure in Figure 6) are insufficient for this task [32]. The antisense oligonucleotide was able to down-regulate cathepsin B expression moderately, albeit significantly and in a reproducible way. Figure 3(B) shows a 30% decrease by the antisense oligonucleotide with respect to the sense control with ELISA detection. A 50% decrease was induced by the antisense oligonucleotide when using the radiolabelling method (Figure 4B). The purpose of this experiment was to verify the feasibility of bridging two non-consecutive exons with an antisense oligonucleotide. Since in preliminary experiments with chemically modified oligonucleotides we observed non-specific cytotoxic effects, to test our hypothesis we preferred a regular, unmodified oligonucleotide despite its lower stability.

DISCUSSION

In this study, we show that transfection of dsRNA or ssDNA encoding cathepsin B inhibits the expression of this enzyme in chondrocytes and thus represents a model for determining the feasibility of using antisense methods and gene silencing to inhibit unwanted proteolysis. Reproducible studies on cathepsin B expression and regulation in chondrocytes have been hampered by the progressive phenotypic changes occurring in cell cultures of normal chondrocytes. Cathepsin B expression was compared in two immortalized human chondrocyte cell lines and normal articular chondrocytes, with the objective of defining a cell line suitable for reproducible studies on enzyme induction, cell transfection and inhibition of cathepsin B expression. Both T/C-

28a2 and C-28/I2 cells exhibited low intracellular levels of cathepsin B and secreted low amounts of this enzyme in a way similar to primary cultures of normal articular chondrocytes. After repeated passaging, the levels remained low compared with subcultured normal chondrocytes artificially dedifferentiated in monolayer culture, which increase enzyme expression, as previously shown by us [10]. Considering cathepsin B storage and secretion, the number of intracellular organelles belonging to the acidic compartment and the distribution of alternatively spliced cathepsin B mRNA forms, the T/C-28a2 cell line very closely resembled chondrocytes freshly isolated from cartilage, and the C-28/I2 cell line was very similar to chondrocytes just starting to dedifferentiate, such as those observed in a primary monolayer culture. Although both lines of immortalized chondrocytes contained discrete amounts of cathepsin B in storage granules, intracellular activity staining, distinguished by deposits of a microcrystalline fluorescent reaction product upon application of a specific detection method, was not observed at all in T/C-28a2 and very rarely in C-28/I2 cells. This property is characteristic of differentiated chondrocytes, as opposed to dedifferentiated cells, which display a characteristic pattern of activity staining [10,11]. We described previously the non-identity of endosomal-lysosomal cathepsin B with the enzyme detectable by activity staining, and identified this last form as a compartment associated with microtubules [9].

The immortalized human chondrocyte cell lines were created as reproducible models for studying gene expression in studies that require large numbers of cells. They have been used successfully to examine expression of cDNAs and reporter genes in various plasmid and viral vectors [33–35]. In the present study, T/C-28a2 cells could be collected as single cell suspension after trypsinization, whereas C-28/I2 cells formed a matrix in which they were embedded and from which they were recovered as aggregates. The *in situ* RT-PCR experiments were performed to visualize the distribution of alternatively spliced cathepsin B mRNAs, and confirmed the clonal nature of C-28/I2 cells, the majority of which were weakly stained. Conversely, T/C-28a2 chondrocytes very much resembled the heterogeneous cell population isolated from articular cartilage, with both positively and negatively stained cells. These experiments and the semi-quantitative determination of the relative abundance of the three cathepsin B mRNAs alternatively spliced in the 5′-UTR were necessary for determining the nature of the two immortalized chondrocyte lines and for comparing their properties with those of normal cells [although in this report we did not further use the information obtained on the CB(−2,3) species]. By virtue of the close resemblance of the T/C-28a2 cell line with normal chondrocytes and the easier handling, it was selected for studies on inhibition of gene expression.

The antisense ssDNA probes used to repress cathepsin B mRNA activity, either containing or not containing exon 2, were all effective, irrespective of their size, which ranged from a nearly full-length sequence to a relatively small fragment of 53 nucleotides in length. Of particular interest was the outcome of a synthetic oligonucleotide with 24 bases, 12 of which hybridized with the end of cathepsin B exon 1 and 12 of which hybridized with the beginning of exon 3. Databank searches demonstrated that this sequence was cathepsin B-specific. Considering that in T/C-28a2 chondrocytes CB(−2) represents a small fraction of the mRNA splice variants differing in the 5′-UTR (Table 2), the inhibitory activity observed with the oligonucleotide bridging exons 1 and 3 suggests a regulatory role for exon 2 in the cathepsin B gene. Namely, the relatively small proportion (5.6 %) of the CB(−2) species lacking exon 2 was responsible for 30–50 % of the total biosynthetic activity. As noted previously [36], alternative splicing of the 5′-UTR represents a possible control mechanism for cathepsin B biosynthesis. In pathological situations such as osteoarthritis [15] and cancer [36] the proportion of cathepsin B mRNA lacking exon 2, as well as cathepsin B biosynthesis, is higher than in the corresponding normal tissues. Thus CB(−2) can be suspected to be at least partly responsible for enzyme overproduction and secretion in the extracellular space. Here we have shown that antisense methods can be specifically addressed to CB(−2) mRNA, resulting in targeted modulation of enzyme biosynthesis.

RNAi is an innovative method for the sequence-specific, post-transcriptional gene silencing through cognate dsRNA. The RNAi mechanism is based on processing dsRNA to small interfering RNA (siRNA) segments of 21–23 nucleotides in length, formation of a protein–siRNA complex, binding of this complex to mRNA and cleavage of the targeted mRNA [37]. This method has been successfully employed to specifically and efficiently inactivate gene activities in invertebrates and plants [38,39]. However, RNAi has been far less effective as a tool for gene silencing in cells from vertebrate organisms [40–42], for instance in mouse oocytes and preimplantation embryos [41], and in hamster ovary cells [42]. Limitations to the use of RNAi in mammalian cells derive from sequence-non-specific responses to long dsRNA, such as induction of interferon synthesis [43] and apoptosis [38]. The recent development of 21-nucleotide siRNA duplexes has circumvented these problems, and allowed successful RNAi in cultures of several types of mammalian cells [44].

In the present study we observed effective silencing of the cathepsin B gene by transfecting immortalized human chondrocytes with dsRNA corresponding to a nearly full-length cDNA. The purpose of this approach was to verify the feasibility of the gene-silencing method in a human cell line and to test any adverse effects resulting from treatment over a relatively short time (4–24 h). Cell viability at the end of the treatments was excellent, as was the cell count and DNA content in comparison with parallel cultures of non-treated cells, and no symptoms of apoptosis were noticed. As a biochemically quantifiable parameter, the interferon cascade was not induced by the relatively long dsRNA used, as demonstrated by the lack of phosphorylation of the α subunit of the general translation initiation factor eIF-2 [30,31,45]. Moreover, transfection with an unrelated dsRNA affected neither cathepsin B biosynthesis nor vital cellular properties. Although we cannot exclude that a treatment for longer periods may possibly induce non-specific side effects, we have shown that human chondrocytes possess the RNAi equipment necessary for exploiting the sequence information contained in the dsRNA for gene-silencing purposes. In conclusion, immortalized human chondrocytes very closely resemble normal articular chondrocytes, and represent a valid model for the application of both classical antisense approaches and RNAi as tools for the suppression of unwanted proteolytic activities.

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