Cloning and characterization of anti-cathepsin L single chain variable fragment whose expression inhibits procathepsin L secretion in human melanoma cells

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We previously demonstrated that increase of procathepsin L secretion by human melanoma cells strongly increased their tumourigenicity and switched their phenotype from low to highly metastatic. Thus, we herein analysed whether it was possible to inhibit procathepsin L secretion using anti-cathepsin L ScFv. For this purpose, we produced different forms of fusion cathepsin L in prokaryotic or eukaryotic expression systems. An anti-cathepsin L monoclonal antibody (mAb), named 3D8, was isolated from mice immunized with purified procathepsin L-His. This 3D8 mAb interacted with an epitope localized on the 156–197 amino acid sequence of cathepsin L and recognized recombinant or native forms of cathepsin L synthesized by human melanoma cells. An active anti-cathepsin L ScFv was

generated and characterized from 3D8 mAb heavy and light variable chains. Then, human melanoma cells were transiently co-transfected with 3D8 ScFv and cathepsin L cDNAs. Data demonstrated that increase of 3D8 ScFv expression in human melanoma cells totally inhibited procathepsin L secretion and induced accumulation of intracellular procathepsin L. Our results constitute the first demonstration that anti-cathepsin L ScFv could be used in human melanoma cells to inhibit procathepsin L secretion. This ScFv represents a new molecular tool to explore cell therapy of human melanomas.

Key words: cell therapy, cysteine protease, third component of complement.

INTRODUCTION

Cathepsin L is a papain-type cysteine proteinase [1–3] whose gene is localized on human chromosome 9q21–22 [4]. Cathepsin L is successively translated as pre-procathepsin L, transferred through the Golgi apparatus as procathepsin L and then stored in lysosomes as mature cathepsin L [5]. Cathepsin L, whose overexpression was demonstrated in transformed cells [6] and human tumours [7], acts as an endopeptidase, which degrades a wide range of intracellular cytoplasmic and nuclear proteins [8,9]. In addition, procathepsin L is also secreted by normal or transformed cells [5] and consequently could control functions of extracellular proteins, by cleavage process. We demonstrated that procathepsin L secreted by human melanoma cells cleaved human C3, the third component of complement, thus inhibiting the cytolytic activity of complement, i.e. controlling the humoral immune system [10,11]. Furthermore, cathepsin L secreted by an endothelial tumour cell line generated endostatin from collagen XVIII, suggesting that cathepsin L could play a role in angiogenesis [12]. Cathepsin L was also involved in tumour progression [13,14]. Indeed, antisense inhibition of cathepsin L mRNA decreased tumour growth of murine myeloma [15]. We demonstrated that pretreatment of tumourigenic and highly metastatic human melanoma cells with anti-cathepsin L antibody (Ab) strongly inhibited their tumourigenicity and significantly decreased their metastatic potential in nude mice [16]. In addition, we demonstrated that increase of procathepsin L secretion by human melanoma cells strongly increased their tumourigenicity and switched their phenotype from non-metastatic to highly metastatic [17]. Indeed, increased secretion of procathepsin L by human melanoma cells ‘arms’ them with the ability to inactivate

complement-mediated cell lysis, thus contributing to tumour growth and metastasis [18]. All these data support the idea that increase of tumourigenic and metastatic properties of human melanoma is related to the increase of procathepsin L secretion.

These results pointed out the need to inhibit secretion of procathepsin L by human melanoma cells. For this reason, it was necessary on one hand to elucidate the molecular mechanisms involved in the regulation of human cathepsin L gene expression or protein secretion and on the other hand to design new molecular tools preventing procathepsin L secretion.

With respect to the first aim, we recently identified molecular elements involved in human cathepsin L transcriptional regulation in melanoma cells [19]. Indeed, we determined the sequence of the 5′-flanking region of human cathepsin L gene up to 3263 bp upstream of the translation start site and localized the major transcription initiation site. The presence of three mRNA splice variants, differing in their 5′-untranslated ends, was demonstrated. Regulatory sites crucial for cathepsin L promoter activity were characterized between –1489 and –1646 bp: in this region two GC boxes and a CCAAT motif were involved in specific DNA–protein interactions. We demonstrated that three transcription factors, Sp1 and Sp3, which bound to GC boxes, and NF-Y, which bound to the CCAAT motif, were essential for human cathepsin L gene transcription in melanoma cells [19].

With respect to the second aim, i.e. the design of new molecular tools which could inhibit procathepsin L secretion, we herein undertook to prepare an anti-human cathepsin L single-chain variable fragment (ScFv). For this purpose, we synthesized different forms of recombinant fusion cathepsin L, one of them being used as an immunogen. An anti-cathepsin L monoclonal

Abbreviations used: Ab, antibody; GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactoside; mAb, monoclonal antibody; ScFv, single-chain variable fragment; VH chain, variable heavy chain; VL chain, variable light chain.

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The nucleotide and deduced amino acid sequences of 3D8 ScFv anti-human cathepsin L have been deposited in the GenBank Nucleotide Sequence database under accession no. AF488378.

antibody (mAb) was isolated and used to generate a ScFv, whose nucleotide and amino acid sequences were determined. The binding properties of this ScFv were analysed on recombinant and native cathepsin L. Then this ScFv was expressed in human melanoma. We herein demonstrate that expression of anti-cathepsin L ScFv in human melanoma cells inhibits procathepsin L secretion.

MATERIALS AND METHODS

Cell lines and culture conditions

Two human melanoma cell lines were used: the DM-4 cell line, established from surgical specimen of lymph node metastasis [20], and the A375SM cell line, established from nude mice lung metastases produced by the A375-P cell line isolated from a lymph node metastasis [21,22]. Both cell lines, highly tumorigenic and metastatic in nude mice, were kindly provided by Dr M. Bar-Eli (M. D. Anderson Cancer Center, Houston, TX, U.S.A.). The P3 × 63Ag8.653 mouse myeloma cell line was obtained from the American Type Culture Collection (CRL-1580). The melanoma and myeloma cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, Glutamax-1, glucose, sodium pyruvate and non-essential amino acids (Invitrogen) at 37 °C in a CO₂ incubator.

Conditioned media and cell-extract preparations

Conditioned media were prepared as described previously [10]. Briefly, DM-4 cells were incubated for 24 h in serum-free culture medium and conditioned media were concentrated 20-fold on a Microcon 10 concentrator (Amicon). Cell extracts were prepared in PBS containing 1% (v/v) Nonidet P-40 and complete protease inhibitor cocktail (Roche). Solubilized proteins were collected by centrifugation and protein concentration was determined by the bicinchoninic acid (BCA) protein assay reagent (Pierce).

Protein analysis

ELISA

To analyse cathepsin L specificity, 96-well plates were coated with 100 µl of glutathione S-transferase (GST) or GST-cathepsin L fusion protein (GST-CTSL) solution at 5 µg/ml and room temperature for 1 h. For antibody production, plates were coated with 100 µl of goat anti-mouse immunoglobins (Dako) at 5 µg/ml. After saturation with 5% low-fat dried milk, for 1 h at room temperature, 100 µl supernatants from hybridoma cells, purified 3D8 mAb or bacterial culture supernatant containing 3D8 ScFv were incubated in microtitre plates for 1 h at room temperature. To detect 3D8 ScFv binding, additional incubation was performed with an anti-E tag antibody at 1 µg/ml (Amersham Biosciences). After three washes, the plates were then probed with a 1:5000 dilution of goat anti-mouse immunoglobins conjugated with horseradish peroxidase (Dako). After three additional washes, the colour reaction was performed by adding *o*-phenylenediamine (0.5 µg/ml) at room temperature for 30 min, followed by the addition of 50 µl of 3 M HCl to stop the colour-development reaction. Antibody binding was determined by measuring absorbance values at 492 nm using a Metertech 960 microplate reader.

Affinity constant determination

Affinity constants of 3D8 mAb and 3D8 ScFv were determined as described by Friguet et al. [23] for mAb and by Bruyns et al.

[24] for ScFv. Briefly, 3D8 mAb or 3D8 ScFv was incubated with different concentrations of C-terminally His-tagged procathepsin L (ProCTSL-His). Then, at the equilibrium state, the concentration of free antibody was determined by ELISA using immobilized ProCTSL-His. The dissociation constants were calculated using the Klotz equation, as detailed [23].

Immunoprecipitation

Prior to immunoprecipitation, 5 µg of purified 3D8 mAb or anti-v-Src mAb, an irrelevant mAb of the same isotype (Ab-1; Calbiochem) was bound to 50 µl of Protein G–Sephacrose beads (Sigma). For experiments with 3D8 ScFv, 50 µl of Protein G–Sephacrose beads was first incubated with 5 µg of anti-E tag mAb and then with 250 µl of bacterial culture supernatant containing 3D8 ScFv. In controls, the anti-E tag mAb was omitted. Then, purified ProCTSL-His (250 ng) or DM-4-cell concentrated conditioned medium (150 µl) was added to immunobeads for 1 h at 4 °C. After extensive washes of immunobeads, proteins were eluted and submitted to SDS/PAGE.

Immunoblotting analysis

Immunoblot assays were performed as described previously [25], using polyclonal anti-cathepsin L antibody (Biogenesis), anti-E tag mAb (Amersham Biosciences), anti-Myc tag mAb (clone 9E10; Upstate Biotechnology), anti-V5 mAb (Invitrogen) or supernatants of 3D8 hybridoma cells. For immunoblotting with 3D8 ScFv, membranes were first incubated with bacterial culture supernatant containing 3D8 ScFv, then probed with anti-E tag mAb. Binding of antibodies was detected with peroxidase-linked secondary antibodies (Dako) and the enhanced chemiluminescence (ECL) system (Amersham Biosciences).

Plasmid constructs

Plasmid pGHP3, corresponding to pGEM3 vector in which procathepsin L cDNA was cloned, was kindly provided by Dr M. M. Gottesman (National Institutes of Health, Bethesda, MD, U.S.A.) [26]. GST-fusion-protein vectors (ProCTSL, procathepsin L; CTSL, cathepsin L; Pro, proregion of cathepsin L) were generated by cloning cDNAs, amplified by PCR from plasmid pGHP3, in the pGEX-4T3 vector (Amersham Biosciences), using the *Bam*HI and *Xho*I sites (underlined below). The 5' primers used were 5'-GCGGATCCCATGACTCTAAC-ATTGATCAC-3' for GST-Pro and GST-ProCTSL and 5'-GCGGATCCCATGGCCCCAGATCTGTGGAT-3' for GST-CTSL. The 3' primers used were 5'-TATCTCGAGTCACAC-AGTGGGGTAGCTGG-3' for GST-CTSL and GST-ProCTSL and 5'-TATCTCGAGTCACTCATAAAACAGAGGTTTC-3' for GST-Pro. His fusion proteins were prepared either in a prokaryotic expression system (His-ProCTSL, His-CTSL and His-Pro), by subcloning cathepsin L cDNAs, cloned into the pGEX-4T3 vector, into the pET-28a(+) vector (Novagen) or in the eukaryotic expression system: pre-procathepsin L cDNA was amplified by PCR from the pGHP3 plasmid with primers 5'-GCGAAGCTTATGAATCCTACACTCATCCT-3' and 5'-GCGGATCCGCACAGTGGGGTAGCTGGCTG-3', and cloned into pTenHisCc (Qbiogen), using the *Hind*III and *Bam*HI sites (underlined), to generate pTenHisCc-ProCTSL.

GST fusion proteins [corresponding to cathepsin L fragments, named pep1–pep5 and detailed in Figure 3 (see below)] used to localize the 3D8 mAb epitope were constructed by cloning PCR products, amplified from pTenHisCc-ProCTSL, in the pGEX-4T3 vector, using the *Bam*HI and *Xho*I sites (underlined below), with the 5' primers 5'-GCGGATCCCATGTATGAGGCCCC-

AGATCT-3' for GST-pep1 (112–295), 5'-GCGGATCCATGGCCCCAGATCTGTGGAT-3' for GST-pep5 (114–156) and GST-pep3 (114–203), 5'-GCGGATCCATGGACTCTGAGGAATCCTAT-3' for GST-pep4 (197–295) and 5'-GCGGATCCATGTTTGAGCCAGACTGTAGC-3' for GST-pep2 (265–334), and the 3' primers 5'-TATCTCGAGATATTTATGTATTATCTGA-3' for GST-pep1 (112–295) and GST-pep4 (197–295), 5'-TATCTCGAGCCAGTTTCCGGAACAT-3' for GST-pep5 (114–156), 5'-TATCTCGAGTCAATAGGATTCCTCAGAGT-3' for GST-pep3 (114–203) and 5'-TATCTCGAGTCACACAGTGGGGTAGCTGG-3' for GST-pep2 (265–334).

The mammalian expression vector encoding a fusion protein of procathepsin L with a C-terminal V5 epitope was generated from pre-procathepsin L cDNA, amplified by PCR from pTenHisCc-ProCTSL vector with primers 5'-GCGAAGCTTAGAATCCTACACTCATCT-3' and 5'-GCGGATCCACAGTGGGGTAGCTGGCTG-3' and cloned into pcDNA3.1/V5-HisB (Invitrogen), using *Hind*III and *Bam*HI (underlined). To allow expression and retention of the 3D8 ScFv in the endoplasmic reticulum of melanoma cells, the 3D8 ScFv cDNA, generated by PCR from pCANTAB5E-3D8/ScFv with primers 5'-ACGCGTCGACATGGCCCAGGTGAAGCTGCAGCAGTTC-3' and 5'-ATAAGAATGCGGCCGCGCCGTTTGATTTCCAGATTGGTGCCAGCACC-3', was then cloned in pCMV/myc/ER (Invitrogen), using *Sall* and *Not*I (underlined). The underlined restriction enzyme site in each primer was added to the 5'-end to facilitate subcloning.

Production of recombinant cathepsin L forms

GST fusion proteins (GST-Pro, GST-CTSL and GST-pep1–GST-pep5) were produced in *E. coli* strain BL21, following the Amersham Biosciences method. GST-Pro and GST-CTSL were purified from soluble and insoluble fractions, respectively. His fusion proteins, with a His tag in the N-terminal position (His-ProCTSL, His-CTSL and His-Pro), were produced using the pET system (Novagen), into *E. coli* strain BL21(DE3) and were purified using the His-bind resin kit (Novagen) from insoluble cell fractions. His fusion protein with a His tag in the C-terminal position, ProCTSL-His was generated using a baculovirus/insect expression system. Co-transfection of Sf9 insect cells with pTenHisCc-ProCTSL vector and baculovirus viral DNA, isolation of recombinant virus, amplification of isolated recombinant clones and large-scale expression of proteins from recombinant baculovirus were performed by Qbiogene. ProCTSL-His was purified using His-bind resin (Novagen) from insect cell-culture supernatant. Purity of the fusion proteins was monitored by SDS/PAGE and protein concentration was determined using the BCA protein assay reagent.

Generation of anti-cathepsin L mAb

Six 7-week-old female BALB/c mice were first subcutaneously immunized with 25 µg of purified fusion procathepsin L (ProCTSL-His). Then, 3 weeks later, mice were intraperitoneally immunized with 25 µg of purified ProCTSL-His in incomplete Freund's adjuvant. This protocol was repeated five times, every 3 weeks. When titre of anti-cathepsin L Ab present in serum was higher than 1/30000 and 3 days after a final boost with 50 µg of purified ProCTSL-His, spleen cells were harvested for fusion, which was performed as described previously [27]. Hybridoma cells surviving selection were analysed by ELISA for antibody production and cathepsin L specificity. Anti-cathepsin L antibody-producing cells were cloned by limiting dilution. The

immunoglobulin class and subclass of the light-chain type of the mAb was determined using the mouse hybridoma subtyping kit (Roche) according to the manufacturer's instructions. mAbs were purified using a Protein G–Sepharose affinity column (Sigma) and antibody concentration was determined using the BCA protein assay reagent.

Preparation of anti-cathepsin L ScFv

Construction of 3D8 ScFv expression vector

Total RNA was isolated from 3D8 hybridoma cells with Trizol (Invitrogen) and subjected to DNase I (Clontech) treatment, then mRNA was isolated using the mRNA-purification kit (Amersham Biosciences). First-strand cDNA synthesis was prepared with random primers using the reverse transcription system kit (Promega). PCR amplifications of the antibody heavy (VH) and light (VL) variable chains were performed using separate reaction mixtures with primers specific for each chain (Amersham Biosciences). Purification of PCR products was performed by gel electrophoresis in agarose gel. The DNA bands of 340 and 325 bp corresponding to VH and VL chains respectively were excised and the DNA was purified by Sephaglas Bandprep Kit (Amersham Biosciences). The VH and VL chains were assembled with a linker by two rounds of PCR amplification as described in the Amersham Recombinant Phage Antibody System (RPAS) kit. The 750 bp DNA band corresponding to the ScFv fragment was purified and cloned into the pCANTAB5E phagemid vector (Amersham Biosciences) using *Sfi*I and *Not*I restriction enzyme sites.

Selection of recombinant phage antibodies specific for cathepsin L

All subsequent steps in the recombinant phage antibody production were carried out with the RPAS (Amersham Biosciences), according to the manufacturer's instructions. Briefly, competent *E. coli* TG1 cells were transformed with pCANTAB5E containing the ScFv fragment. Individual transformed colonies were infected with M13KO7 phage to rescue the phagemid. Recombinant phage antibodies, which arose from each clone, were analysed for cathepsin L binding activity in an ELISA assay. Microtitre plates were coated with 100 µl of GST-CTSL solution at 10 µg/ml and recombinant phage antibodies were added. Phage binding was detected with a peroxidase-labelled sheep anti-M13 antibody (Amersham Biosciences). Plates coated with GST were used as a negative control. Among 48 recombinant phage clones analysed by this assay, four clones bound specifically to cathepsin L. The recombinant phage clone, which gave the strongest positive signal in ELISA, was used to infect *E. coli* HB2151 cells.

Expression of the anti-cathepsin L ScFv in *E. coli* HB2151 cells

After infection with the recombinant phage antibodies specific for cathepsin L, individual HB2151 colonies were transferred to 5 ml of 2 × YT medium containing 100 µg/ml ampicillin and 1 % (w/v) glucose and incubated overnight at 37 °C. Aliquots of 100 µl of cell suspension were grown in 2 ml of 2 × YT medium containing 100 µg/ml ampicillin and 0.1 % (w/v) glucose until cell culture reached an attenuation of 0.5 or 1 at 600 nm. Cells were induced overnight with 0.2–1 mM isopropyl β-D-thiogalactoside (IPTG), in presence or absence of 0.1 % glucose, at 16, 24 or 30 °C. Cell lysate and culture supernatant were analysed for the presence of soluble ScFv fragments by immunoblot assay using the anti-E tag mAb, specific to the peptide E tag located at the C-terminus of the ScFv molecule (Amersham Biosciences). ScFv secretion was estimated by analysing ScFv protein band intensity using the public-domain NIH Image

program. Plasmid pCANTAB5E-3D8/ScFv was isolated from HB2151 cells and the DNA region encoding 3D8 ScFv was sequenced by Genome Express (Montreuil, France).

Confocal microscopy analysis

Cells growing exponentially on glass slides were fixed in cold 100% methanol for 5 min and then permeabilized in PBS containing 0.2% (v/v) Triton X-100 for 10 min at room temperature. After saturation, in PBS containing 0.2% BSA for 45 min, cells were incubated with 3D8 mAb (10 µg/ml) for 1 h at room temperature. For experiments with 3D8 ScFv, the cells were first incubated with bacterial culture supernatant containing 3D8 ScFv and then with anti-E tag mAb (10 µg/ml). After washing, the cells were then incubated with Alexa Fluor 546 goat anti-mouse IgG (H+L) coupled to phycoerythrin (Molecular Probes), for 1 h at room temperature. Washed cells were embedded in Prolong Antifade mounting medium (Molecular Probes). Computer-assisted image analysis of fluorescence was performed using a confocal scanning laser microscope (Leica TCS).

Transient cell transfections

A375SM melanoma cells were transfected as described previously [19]. Transfections were performed with the indicated amount of vector constructs and, in experiments with increasing amount of vector, the molar equivalent of parent vector was added (without cDNA insert) to maintain an equal amount of vector in each transfection. After transfection (24 h), medium was replaced for 24 h by serum-free medium to generate conditioned medium. Cell extracts were prepared 48 h after transfection.

RESULTS AND DISCUSSION

Preparation and characterization of anti-cathepsin L mAb

In order to generate and to characterize specific anti-cathepsin L mAb, several recombinant forms of cathepsin L were prepared

corresponding to cathepsin L, procathepsin L and the proregion alone of procathepsin L. For this purpose, different systems of fusion protein expression were used (Figure 1). GST (Figure 1A) or His (Figure 1B) fusion proteins were produced in prokaryotic cells. His fusion protein was also generated using a baculovirus/insect expression system (Figure 1C). The limit of protein production of each system used and the difference in the yield of purification of these fusion proteins led us to use mainly the GST-CTSL and GST-Pro forms and the ProCTSL-His form, this latter being produced on a large scale and purified from eukaryotic cells. Purified ProCTSL-His (Figure 1C, lane 3) was used to immunize mice. Antibody specificity and titre in the mice serum was determined by ELISA and immunoblotting assay using purified GST-CTSL. When the titre of anti-cathepsin L Ab present in serum reached the expected high titre, mouse spleen cells were fused to obtain hybridoma. Three weeks after cell fusion, hypoxanthine/aminopterin/thymidine selective medium allowed us to select 113 hybridomas. These hybridomas were tested by ELISA for antibody production and cathepsin L specificity. Only one hybridoma, the 3D8, was identified as an anti-human cathepsin L Ab cell producer. This 3D8 hybridoma was cloned by limited dilution. The difference observed between the high titre of anti-human cathepsin L antibodies in immunized mice and the low yield of selected positive anti-human cathepsin L hybridoma is more likely due to a possible interference of anti-cathepsin L Ab with the functions of endogenous cathepsin L, which is known to play an important role in hybridoma growth. Indeed, it was demonstrated by others that hybridoma cells producing an anti-cathepsin L mAb lost their property to induce solid tumours after implantation into mice [28]. In addition, inhibition of cathepsin L expression also decreased tumour growth of murine myeloma, which were used for fusion with spleen cells [15]. These observations suggested that the selected 3D8 mAb is an anti-cathepsin L mAb, which did not modify the function of procathepsin L involved in cell growth. This is also supported by the demonstration that 3D8 mAb, while strongly interacting with procathepsin L (K_d , 6×10^{-9} M), did not inhibit the C3-cleaving activity of this proteinase (results not shown), a

Figure 1 Preparation of cathepsin L fusion proteins

(A) Purified GST fusion proteins were separated by SDS/PAGE and stained with Coomassie Blue. Lane 1, GST-Pro (3.3 µg); lane 2, GST-CTSL (1.8 µg); lane 3, GST (9 µg). (B) Purified His fusion proteins, produced in prokaryotic cells, were separated by SDS/PAGE and stained with Coomassie Blue. Lane 1, His-Pro; lane 2, His-CTSL; lane 3, His-ProCTSL. (C) Culture supernatant from Sf9 insect cells, after transfection without (lane 1) or with (lane 2) procathepsin L recombinant viral DNA, was submitted to SDS/PAGE and subsequently immunoblotted with the polyclonal anti-cathepsin L Ab. Purified ProCTSL-His (1 µg), generated using a baculovirus/insect expression system, was submitted to SDS/PAGE and stained with Coomassie Blue (lane 3).

Figure 2 Characterization of anti-cathepsin L 3D8 mAb

(A) ELISA with purified 3D8 mAb using recombinant GST or GST-CTSL proteins. (B) Immunoblot of purified fusion cathepsin L proteins (100 ng) using 3D8 mAb. Lane 1, ProCTSL-His; lane 2, GST-Pro; lane 3, GST-CTSL. (C) Immunoprecipitation of ProCTSL-His by anti-v-Src mAb (lane 1) or by anti-cathepsin L 3D8 mAb (lane 2). Immunoprecipitated proteins were submitted to SDS/PAGE, then immunoblotted with polyclonal anti-CTSL Ab. (D) Immunoblot using 3D8 mAb (lanes 1 and 2) or polyclonal anti-cathepsin L Ab (lanes 3 and 4) on conditioned media (lanes 1 and 3) or cell extracts (lanes 2 and 4) prepared from DM-4 cells. (E) Immunoprecipitation from DM-4 cells conditioned media using anti-v-Src mAb (lane 1) or by 3D8 anti-cathepsin L mAb (lane 2). Immunoprecipitated proteins were submitted to SDS/PAGE, then revealed by polyclonal anti-cathepsin L Ab. (F) Confocal microscopy analysis of DM-4 melanoma cells stained without (panel 1) or with (panel 2) 3D8 mAb.

specific activity that we previously demonstrated [16,17]. Further studies are needed to analyse the reasons for this difference and the biological significance of this selection.

Isotype analysis demonstrated that 3D8 mAb was an IgG1 with a κ light-chain type. Characterization of 3D8 specificity was performed. As shown in Figure 2(A), 3D8 mAb recognized GST-CTSL but not GST in ELISA. In addition (Figure 2B), 3D8 reacted with ProCTSL-His (lane 1) or GST-CTSL (lane 3) but not GST-Pro (lane 2) in the immunoblotting assay, and (Figure

2C) immunoprecipitated ProCTSL-His (lane 2). An anti-v-Src mAb, with the same IgG1 isotype, was used as negative control (Figure 2C, lane 1). The specificity of 3D8 mAb was also analysed on native forms of cathepsin L expressed by DM-4 human melanoma cells; i.e. the 41 kDa procatepsin L secreted by these cells and the two 34 and 29 kDa mature forms present in cell extracts [11,17]. The 3D8 anti-cathepsin L mAb recognized (Figure 2D) the 41 kDa procatepsin L in conditioned media (lane 1) and in cell extracts (lane 2), and the 29 and 34 kDa mature forms of cathepsin L present only in cell extracts (lane 2). A polyclonal anti-cathepsin L Ab was used as positive control (lanes 3 and 4). Furthermore, 3D8 mAb specifically immunoprecipitated procatepsin L from DM-4 cell conditioned media (Figure 2E, lane 2). 3D8 mAb also recognized cathepsin L *in situ*, as demonstrated by specific positive immunofluorescent staining observed by confocal microscopy of permeabilized DM-4 melanoma cells (Figure 2F).

In addition, we determined the domain of cathepsin L recognized by 3D8 mAb. For this purpose, GST fusion proteins corresponding to several cathepsin L fragments were prepared. Specificities of 3D8 mAb were tested by immunoblot on the fusion peptide produced from each construct (Figure 3). Polyclonal anti-cathepsin L, which recognized all constructs except GST-Pro, was used as a control. 3D8 mAb recognized GST-CTSL (114–334), GST-pep1 (112–295) and GST-pep3 (114–203) but not GST-pep2 (265–334), GST-pep4 (197–295) or GST-pep5 (114–156). These results demonstrated that 3D8 mAb recognized recombinant, as well as the 29, 34 and 41 kDa native forms of cathepsin L expressed in human melanoma cell lines, by interacting with an epitope localized on the 156–197 amino acid sequence of this proteinase. The property of 3D8 to interact with procatepsin L, without modifying its C3-cleaving activity (as already mentioned above), suggested that the 156–197 epitope of procatepsin L was not involved in this catalytic function of procatepsin L.

Preparation and characterization of anti-cathepsin L ScFv from 3D8 mAb

Genes of the VH and VL chains of 3D8 mAb present in the hybridoma cells were amplified by two separate PCR reactions from cDNAs, using primers specific to opposite ends of the variable region of each chain (Figure 4A): these amplifications yielded DNA fragments of approx. 340 bp for VH (lane 2) and 325 bp for VL (lane 3). The 3D8 ScFv DNA fragment, generated from these purified VH and VL DNA products linked into a single gene using a DNA linker fragment, was characterized by a length of approx. 750 bp (lane 4). This ScFv DNA fragment was cloned into the phagemid pCANTAB5E and transformed into *E. coli* TG1 cells, which allowed production of phage-displayed recombinant ScFv, as described in the Materials and methods section. Phages, which gave a positive signal in ELISA for binding to cathepsin L via ScFv displayed on their tips, were used to infect *E. coli* HB2151 cells for production of soluble recombinant ScFv. The expression of 3D8 ScFv by HB2151 cells activated by IPTG was monitored by SDS/PAGE and immunoblot analysis using mAb directed against E tag peptide fused to the 3D8 ScFv in the phagemid pCANTAB5E (Figure 4B). 3D8 ScFv, characterized by a molecular mass of 28 kDa, was expressed in the cell lysates (lane 1) and secreted into the bacterial culture supernatant (lane 2).

The optimal conditions for higher secretion of 3D8 ScFv by bacterial cells were established (Table 1). (a) A bacterial cell density of $D_{600} = 0.5$ –1 and 0.2–1 mM IPTG did not significantly

Figure 3 Localization of cathepsin L epitope recognized by 3D8 anti-cathepsin L mAb

Schematic representation of 3D8 anti-cathepsin L mAb and polyclonal anti-cathepsin L Ab reactivities with ProCTSL-His and GST fusion proteins corresponding to fragments of procathepsin L, as determined by immunoblot analysis.

Figure 4 Preparation of 3D8 anti-cathepsin L ScFv

(A) Ethidium-bromide-stained agarose gel separation of the purified DNA fragments corresponding to VL (lane 2) and VH (lane 3) chains of 3D8 mAb and of the assembled ScFv DNA (lane 4). DNA molecular size standards are shown in lanes 1 and 5. (B) Immunoblot of cell lysates (lane 1) and culture supernatant (lane 2) of *E. coli* HB2151 clone, infected by phage-displayed recombinant ScFv specific for cathepsin L, with anti-E tag mAb.

modify ScFv quantity in bacterial culture supernatant. (b) The absence of glucose in bacterial culture medium during IPTG induction increased the ScFv secretion by 17 times; the results supported the contention that glucose inhibited ScFv secretion. (c) ScFv secretion induced by IPTG was increased at room temperature, when compared with other temperatures tested. Then, the 3D8 ScFv produced, using the above optimal conditions, was analysed and characterized for its reactivity with human cathepsin L. Nucleotide and deduced amino acid sequences of VH and VL 3D8 ScFv are shown in Figure 5.

Table 1 The optimal conditions for higher secretion of 3D8 ScFv by bacterial cells

ScFv secretion was estimated from the Western blot with anti-E tag antibody using the public-domain NIH Image program. The ScFv secretion obtained after induction of bacterial cells ($D_{600} = 1$) with 1 mM IPTG in the presence of glucose (0.1%) at 30 °C was set to 1.

IPTG (mM)	Cell density (D_{600})	Glucose (% w/v)	Temperature (°C)	ScFv secretion
0.2	1	0.1	30	0.8
0.5	1	0.1	30	1.1
1	1	0.1	30	1
1	0.5	0.1	30	0.8
1	1	0	30	17.4
1	1	0	16	9.4
1	1	0	24	24.9

We analysed these sequences using several databases. Sequence analysis using the mouse Ig database (<http://www.dnablot.de>) revealed that the 3D8 VH belongs to the VHI family and that the 3D8 VL belongs to the VK 4/5 Group IV subgroup and allowed us to localize the complementarity-determining regions (CDRs; Figure 5). In addition, GenBank analysis using BLAST sequence-alignment software revealed that VH and VL sequences shared 97% homology with several previously described mouse immunoglobulin variable-chain sequences (GenBank accession nos. X59180 for VH, and L37409, U12579 and M94023 for VL). Although these variable chains were part of different specificities, our data demonstrated that the association of VH and VL described herein led to cathepsin L specificity.

VH

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1          10          20
GCC CAG GTG AAG CTG CAG CAG TCT GGG TCT GAG CTG GTG AGG CCT GGA GCT TCA GTG AAG
A  Q  V  K  L  Q  Q  S  G  S  E  L  V  R  P  G  A  S  V  K

21          30          40
CTG TCC TGC AAG GCT TCT GGC TAC ACA TTC ACC AGC TAC TGG ATG CAC TGG GTG AAG CAG
L  S  C  K  A  S  G  Y  T  F  T  S  Y  W  M  H  W  V  K  Q
                        CDR 1

41          50          60
AGG CAT GGA CAA GGC CTT GAG TGG ATT GGA AAT ATC TAT CCT GGT AGT GGT AAT ATT AAT
R  H  G  Q  G  L  E  W  I  G  N  I  Y  P  G  S  G  N  I  N
                        CDR 2

61          70          80
TAT GAT GAG AAG TTC AAG AGC AAG GGC ACA CTG ACT GTA GAC ACA TCC TCC AGC ACA GCC
Y  D  E  K  F  K  S  K  G  T  L  T  V  D  T  S  S  S  T  A

81          90          100
TAC ATG CAC CTC AAC AGG CTG ACA TCT GAG GAC TCT GGG GTC TAT TAC TGT AGA GTC TAC
Y  M  H  L  N  R  L  T  S  E  D  S  G  V  Y  Y  C  R  V  Y
                        CDR 3

101          110
GGT AGG AAC TAC GAA CGG TAC TTC GAT GTC
G  R  N  Y  E  R  Y  F  D  V

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VL

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1          10          20
GCA ATC AAG TCT GCA TCT CCA GGG GAT AAG GTC ACC ATA ACC TGC AGT GCC AGC TCA AGT
A  I  K  S  A  S  P  G  D  K  V  T  I  T  C  S  A  S  S

21          30          40
GTA AAT TAC ATG CAC TGG TTC CAG CAG AAG CCA GGC ACT TCT CCC AAA CTC TGG ATT TAT
Y  N  Y  M  H  W  F  Q  Q  K  P  G  T  S  P  K  L  W  I  Y
                        CDR 1

41          50          60
AGT ACG TCC GAC CTG GCT TCT GGA GTC CCT GCT CGC TTC AGT GGC AGT GGA TCT GGG ACC
S  T  S  D  L  A  S  G  V  P  A  R  F  S  G  S  G  S  G  T
                        CDR 2

61          70          80
TCT TAC TCT CTC ACA ATC AGC CGA ATG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG
S  Y  S  L  T  I  S  R  M  E  A  E  D  A  A  T  Y  Y  C  Q

81          90
CAA AGG AGT AGT TAC CCA CTC ACG TTC GGT GCT GGC ACC AAG CTG GAA ATC AAA CGG
Q  R  S  S  Y  P  L  T  F  G  A  G  T  K  L  E  I  K  R
                        CDR 3

```

Figure 5 Nucleotide and deduced amino acid sequences of the VH and VL regions of 3D8 ScFv

Complementarity-determining regions (CDRs) 1, 2 and 3 are underlined.

The immunoreactivity of the soluble 3D8 ScFv was analysed using recombinant cathepsin L by ELISA, immunoblot and immunoprecipitation assays. As shown in Figure 6, 3D8 ScFv interacted with purified GST-CTSL but not with GST using ELISA (Figure 6A). Immunoblotting assays (Figure 6B) confirmed that 3D8 ScFv interacted with GST-CTSL (lane 3) and His-CTSL (lane 5), but not with GST (lane 1), GST-Pro (lane 2) or His-Pro (lane 4). In addition, 3D8 ScFv immunoprecipitated ProCTSL-His (Figure 6C, lane 2). The interactions of 3D8 ScFv and 3D8 mAb with ProCTSL-His were compared: the dissociation constant of the 3D8 ScFv (5×10^{-8} M) was 10-fold lower than that of 3D8 mAb (6×10^{-9} M). Despite this difference, more likely due to the structural differences between ScFv and mAb, confocal microscopy analysis demonstrated that 3D8 ScFv still recognized cathepsin L *in situ* in melanoma cells (Figure 6D). Thus, 3D8 ScFv interacts also with intracellular cathepsin L.

3D8 ScFv inhibits procathepsin L secretion from human melanoma cells

To analyse the effect of 3D8 ScFv on procathepsin L secretion from human melanoma cells, A375SM cells were co-transfected with the pCMV/myc/ER-3D8/ScFv and pcDNA3.1/V5-His-ProCTSL vectors. The pCMV/myc/ER-3D8/ScFv vector was prepared by subcloning cDNA of 3D8 ScFv into the pCMV/myc/ER vector. This vector targets and retains a protein in the endoplasmic reticulum, the subcellular compartment where procathepsin L is normally translated before being carried to lysosomes or secreted [5]. The 3D8 ScFv was therefore expressed as a fusion protein with a N-terminal signal peptide, an endoplasmic reticulum retention signal (peptide SEKDEL) and the Myc epitope at the C-terminus, which added 2 kDa to the 3D8 ScFv. The pcDNA3.1/V5-His-ProCTSL vector encodes a fusion

Figure 6 Characterization of 3D8 anti-cathepsin L ScFv

(A) ELISA using 3D8 ScFv on GST or GST-CTSL. (B) Immunoblot analysis of purified fusion cathepsin L proteins using 3D8 ScFv. Lane 1, 100 ng of GST; lane 2, 100 ng of GST-Pro; lane 3, 100 ng of GST-CTSL; lane 4, 100 ng of His-Pro; lane 5, 200 ng of His-CTSL. (C) Immunoprecipitation of ProCTSL-His by 3D8 ScFv without (lane 1) or with (lane 2) anti-E tag mAb. After separation by SDS/PAGE, proteins were immunodetected with polyclonal anti-cathepsin L Ab. (D) Confocal microscopy analysis of DM-4 melanoma cells stained without (panel 1) or with (panel 2) 3D8 ScFv, both revealed with anti-E tag mAb.

procathepsin L with a C-terminal V5 epitope (ProCTSL-V5), which allowed protein detection with an anti-V5 mAb. After 48 h co-transfection, conditioned media and cell extracts were prepared from transfected A375SM cells and analysed by immunoblot. Data demonstrated that 3D8 ScFv was expressed in these human melanoma cells (Figure 7A). Indeed, anti-Myc mAb detected the ScFv characterized, as expected, by a molecular mass of 30 kDa in cell extracts prepared from A375SM cells transfected with the pCMV/myc/ER-3D8/ScFv vector (lane 2) but not in cell extracts from A375SM cells transfected with the control pCMV/myc/ER vector (lane 1). In addition, expression of ectopic procathepsin L, characterized as 44 kDa, was analysed in conditioned media and cell extracts prepared from transfected A375SM cells, by immunoblot using anti-V5 mAb. Results demonstrated that: (a) (Figure 7B, lanes 2–7) in the conditioned media, expression of the 44 kDa tagged procathepsin L strongly decreased when pCMV/myc/ER-3D8/ScFv concentrations were increased from 0 to 0.5 μ g, and was almost totally inhibited by 1 μ g; (b) (Figure 7C, lanes 2–5) in cell extracts, the amount of the 44 kDa tagged procathepsin L increased, following opposite kinetics to those measured in extracellular media, suggesting accumulation of ProCTSL-V5 in the endoplasmic reticulum. Altogether, these data demonstrated strongly that expression of 3D8 ScFv anti-cathepsin L in human melanoma cells inhibited procathepsin L secretion. In addition, preliminary studies, using

Figure 7 Intracellular expression of 3D8 ScFv anti-cathepsin L inhibits procathepsin L secretion

(A) Immunoblot of extracts of A375SM cells transfected with 1 μ g of pCMV/myc/ER (lane 1) or pCMV/myc/ER-3D8/ScFv (lane 2) with anti-Myc mAb. (B) Immunoblot of conditioned media of A375SM cells transfected with pCMV/myc/ER-3D8/ScFv alone (lane 1), pcDNA3.1/V5-His-ProCTSL alone (lane 2) and pcDNA3.1/V5-His-ProCTSL with increasing concentrations of pCMV/myc/ER-3D8/ScFv (lanes 3–7) with anti-V5 mAb. (C) Immunoblot using anti-V5 mAb of extracts of A375SM cells transfected with pCMV/myc/ER-3D8/ScFv alone (lane 1), pcDNA3.1/V5-His-ProCTSL alone (lane 2) and pcDNA3.1/V5-His-ProCTSL with increasing concentrations of pCMV/myc/ER-3D8/ScFv (lanes 3–5).

the complement lysis assays that we described previously [10,16], showed that 3D8 ScFv expression in melanoma cells led to a 50% increase of their susceptibility to complement lysis. This increased percentage is close to that (60%) which we previously measured with melanoma cells preincubated with polyclonal anti-cathepsin L Ab, which fully inhibited activity of secreted procathepsin L [10]. Further studies are needed to elucidate the biological functions of this 3D8 ScFv, on human melanoma cells secreting procathepsin L.

In conclusion, our data constitute the first demonstration that anti-cathepsin L ScFv expressed in human melanoma strongly inhibits procathepsin L secretion. The next step of this study is to select human melanoma cells stably transfected with this 3D8 ScFv construct. These cells will be very helpful to analyse whether 3D8 ScFv may be used as a new molecular tool to inhibit tumourigenic and metastatic phenotype of human melanoma cells.

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