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Demonstration of cathepsins B, H and L in xenografts of normal and Duchenne-muscular-dystrophy muscles transplanted into nude mice

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The activities and contents of the lysosomal cysteine proteinases cathepsins B, H and L were examined in xenografts of biopsied muscles transplanted from age-matched normal subjects and Duchenne-muscular-dystrophy (DMD) patients into nude mice. The activity of cathepsin B increased 9-fold and that of B-plus-L increased 24-fold in the first week after transplantation in normal muscle xenografts. By the third week, the activity of cathepsin B increased a total of 20-fold and B-plus-L increased to 36-fold the original level. The activity levels of cathepsin B, B-plus-L, H and D, and acid phosphatase in normal and DMD xenografts were not significantly different when compared 2 weeks after transplantation. However, the protein content of cathepsin B in DMD muscle xenografts was more than 3-fold that of normal xenografts at 2 weeks. The profile of cathepsin H activity in normal muscle xenografts was different than those of cathepsins B and B-plus-L. In the first week, the cathepsin H diminished sharply to about one-third of the biopsied muscle level and then, by 3 weeks after transplantation, it had increased slightly to about half the original level. The amount of endogenous cysteine-proteinase inhibitor changed in parallel with the activity of cathepsins B and B-plus-L. Cathepsins B and H, but not cathepsin L, were found immunohistochemically in regenerating muscle fibres of normal and DMD xenografts 2 weeks after transplantation. Staining of cathepsin B in DMD xenografts was slightly stronger than that in normal subjects. There was no immunostaining in degenerating or necrotic muscle fibres 2 weeks after transplantation. Western-blot analysis revealed that the cathepsin B band at 29 kDa was increased in normal xenografts 2 and 3 weeks after transplantation. Also, 2 weeks after transplantation the staining intensity of this band was slightly stronger in DMD xenografts than in normal xenografts. These results suggest that cathepsin B participates in the regeneration of transplanted muscle, both normal and DMD, and in the DMD muscle fibre-wasting processes, during regeneration.

INTRODUCTION

Roles of the lysosomal acid cysteine proteinases cathepsins B and L in the pathological processes of inflammation [1,2] and tumour invasion or metastasis [3–5] have been described. Studies of the involvement of lysosomal cathepsin B, H, L in the pathogenesis of muscle wasting diseases have been described in detail [6]. The muscular disease Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive genetic disorder in the DMD locus of boys in which a protein product, dystrophin, is lacking in sarcolemma of skeletal muscle [7–15]. Muscle regeneration in DMD is evident in the early stages of the disease [16–21], but there is a net loss of muscle fibrous proteins, which suggests that the reduction in muscle proteins is due to excess protein catabolism. Several investigators have described increases of some proteolytic enzymes in biopsied muscle from patients with DMD [22–24], but only cathepsin B increased markedly in the early stages of the disease [25]. Recently, Kominami *et al.* [26] immunohistochemically demonstrated cathepsins B and H in some hyaline fibres, but there was no reaction in the centronucleated muscle fibres in skeletal muscle from patients with DMD. These findings suggest that both cathepsins may be involved in the degradation of damaged muscle fibrous proteins and removal of these proteins from the muscle cells. Lysosomal systems stained positively with acid phosphatase have been demonstrated in regenerating skeletal muscle cells [27], but there has been no information about the lysosomal acid cysteine proteinases cathepsins B, H and L in muscle xenografts from normal subjects and patients with DMD transplanted into nude mice.

In the present paper we report the activities and contents of cathepsins B, H and L, as well as the amounts of their endogenous inhibitors, in normal and DMD muscle xenografts at weekly intervals after transplantation into nude mice. Cathepsins B, H and L in muscle xenografts were also assessed by Western-Blot analysis, and the predominant presence of cathepsin B was immunohistochemically demonstrated in normal and DMD regenerating muscle cells.

EXPERIMENTAL

Materials

Carbobenzoxy-Phe-Arg-4-methylcoumaryl-7-amide (Z-Phe-Arg-MCA), Z-Arg-Arg-MCA and Arg-MCA were purchased from Peptide Institute (Osaka, Japan). N^{α}-benzoyl-DL-arginine 2-naphthylamide (Bz-Arg-Nap), bovine haemoglobin (Type II) and papain (Type IV) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *p*-Nitrophenyl phosphate was from Wako Pure Chemical (Osaka, Japan). Human cathepsins B and H were purified from autopsied liver homogenate by the method

Abbreviations used: DMD, Duchenne muscular dystrophy; CPI, cysteine-proteinase inhibitor; Z-, carbobenzoxy-; MCA, 4-methylcoumaryl-7-amide; Bz-Arg-Nap, N^{α}-benzoyl-DL-arginine 2-naphthylamide; HRP, horseradish peroxidase; PBS, Dulbecco's phosphate-buffered saline.

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of Schwartz & Barrett [28], and human cathepsin L by the method of Mason *et al.* [29]. Antisera and IgGs against each cathepsin were prepared by methods described previously [30]. Anti-cathepsin IgGs were conjugated with horseradish peroxidase (HRP; Sigma, Type IV) by the method of Nakane & Kawaoi [31], and the conjugates, in Dulbecco's phosphate-buffered saline (PBS; Nissui Pharmaceutical Co.) containing 0.01% NaN_3, were stored at −80 °C until used.

Muscle specimens

Quadriceps femoris muscle biopsies were obtained from eight boys aged 3–11 years (mean 6.4 years) who had DMD. All boys had the characteristic clinical manifestations with high serum creatine kinase levels, myopathic electromyographic change and typical dystrophic muscle pathology. For controls, histochemically normal human quadriceps femoris muscle biopsies were obtained from eleven age-matched patients undergoing orthopaedic operations. Transplantation of muscle specimens into the nude mice was performed by the procedure described by Wakayama *et al.* [32,33]. Briefly, recipient nude mice were anaesthetized with pentobarbital, and two small incisions were made in the skin of the right and left back, 1–2 mm from the paravertebral musculature. The biopsied muscle tissue was immediately placed in sterile PBS and divided into pieces 2–3 mm in diameter by 5–6 mm long (average 12 mg wet weight/piece). One such piece was frozen in liquid nitrogen for analysis at zero time (0 weeks). The others were inserted, one into each incision, and were not in direct physical contact with the host muscle. The incisions were closed with cotton sutures. All procedures were done under sterile conditions within 2–3 h. The recipient animals were killed 1, 2 and 3 weeks after the transplantation, and the subcutaneous space in the back was opened. The skin bearing the grafted tissue was stretched with pins. The muscle grafts were dissected from the surrounding fibrous and subcutaneous connective tissue. Muscle samples were immediately frozen in isopentane cooled by liquid nitrogen and stored at −80 °C until assay. For each biopsied muscle, two or three nude mice were used for the transplantation following the above procedure. At least four muscle grafts were examined in the following experiments after each interval.

Preparation of muscle extracts

The stored muscle samples were homogenized in 50 vol. of 20 mM-sodium phosphate, pH 6/0.1% Triton X-100 with a ground-glass homogenizer. The soluble extracts (average 1.15 ml/mouse) were obtained by centrifuging (11000 *g*, 20 min, 4 °C) in a Hitachi CR20B2 centrifuge. Portions of the extracts were used for assaying enzyme activity and content, and amount of endogenous cysteine-proteinase inhibitor (CPI). Protein concentration of each extract was measured by the method of Lowry *et al.* [34], with BSA as a standard.

Measurements of enzyme activity and amount of CPI

Cathepsins B and H activities were assayed with Z-Arg-Arg-MCA at pH 6.0 and Arg-MCA at pH 6.8 respectively by measuring MCA liberation [35]. Cathepsin L activity was assayed with Z-Phe-Arg-MCA at pH 5.5 [35]. Since Z-Phe-Arg-MCA is hydrolysed not only by cathepsin L but also cathepsin B at pH 5.5, its hydrolysis is expressed as the activity of cathepsin B-plus-L. Cathepsin D was assayed by the method of Barrett [36], and acid phosphatase was assayed by the method of Igarashi & Hollander [37]. The amount of CPI was measured by the method of Sano *et al.* [38], in which 150 μl of extract was adjusted to about pH 2.0 with HCl, heated in a boiling-water bath for 1 min, adjusted to pH 8.0 with NaOH and the supernatant was obtained by centrifugation at 11000 *g* for 10 min at 4 °C. Papain activity was assayed as Bz-Arg-Nap hydrolysis. A unit of inhibitor was defined as the amount causing 1 unit decrease in papain activity. Enzyme activity and the amount of CPI of the transplanted normal xenografts were measured 1, 2 and 3 weeks after transplantation. The enzyme activity and CPI of DMD muscle xenografts were analysed at 2 weeks, because of the small amount of biopsied DMD muscle specimens available for this study.

Measurement of cathepsin content by e.l.i.s.a.

The contents of cathepsins B, H and L in normal and DMD muscle xenografts were measured 2 weeks after transplantation by e.l.i.s.a. with a microtitre plate as follows: Wells of an irradiated microtitre plate (96 wells; Dynatech Immulon, Alexandria, VA, U.S.A.) were coated with 100 μl of affinity-purified IgGs (13 μg/ml in 0.1 M-carbonate buffer, pH 9.6) with monospecific reactivity against cathepsins. The plate was sealed and left overnight at 4 °C. After removal of the IgG solution, the wells were washed three times with PBS/0.05% Tween-20 (PBS/Tween). Then 100 μl of 3:1- or 5:1-diluted muscle extracts or purified cathepsins (0.7–20 ng/well) as standards in PBS/Tween/1% BSA, were applied to each well. The plate was sealed and left for 1 h at 37 °C. The wells were washed three times with PBS/Tween, and 100 μl of IgG–HRP conjugates (190 ng/ml) diluted in PBS/Tween/0.1% BSA was added to each well. The plate was incubated for 30 min at 37 °C. After removal of the conjugate and washing three times with PBS/Tween, 100 μl of 0.04% *o*-phenylenediamine/1.82 mM-H_2O_2 in citrate/phosphate buffer, pH 5.0, was quickly added to each well before the plates were placed in the dark. The enzymic reaction was stopped after 30 min by the addition of 25 μl of 10 M-H_2SO_4. The absorbance of each well was recorded at 492 nm by an automated e.l.i.s.a. reader (Bio-Rad).

Western-blotting analysis

SDS/PAGE was performed by the method of Laemmli [39], with 12.5% gel. The proteins were blotted on to nitrocellulose by the method of Towbin *et al.* [40] at 30 V for 3 h. The blots were soaked in PBS/3% BSA and then incubated with 1:600 dilutions of anti-(cathepsins B), anti-(cathepsin H) and anti-(cathepsin L) sera. The protein bands were detected by a colour reaction with the ProtoBlot immunoblotting system (Promega Biotec, Madison, WI, U.S.A.).

Histochemical and immunohistochemical methods

Serial frozen sections (7 μm thick) were placed on glass slides and stained with both haematoxylin/eosin and Acridine Orange stain [41] to identify any regenerating muscle fibres. For immunohistochemical examination, sections were immersed in 100% methanol/0.3% H_2O_2 for 30 min at room temperature and then incubated at room temperature for 20 min with 5% normal rabbit serum diluted in PBS. After washing three times in PBS, they were incubated overnight at 4 °C with rabbit anti-(human cathepsins B, H and L)-IgG–HRP conjugates diluted 1:20 in PBS and washed three times in PBS. The sections were stained with 0.04% 3,3′-diaminobenzidine/0.01% H_2O_2 in PBS for 5 min at room temperature, dehydrated, cleared, and mounted. Control sections were incubated with preimmune serum.

RESULTS

The activities of cathepsins B, B plus L, and H, and the amount of CPI in normal muscle xenografts 1, 2 and 3 weeks after transplantation are shown in Fig. 1. The activities of cathepsins B and B plus L increased dramatically the first week after transplantation; cathepsin B increased to 4.89 ± 0.97 μmol/h per

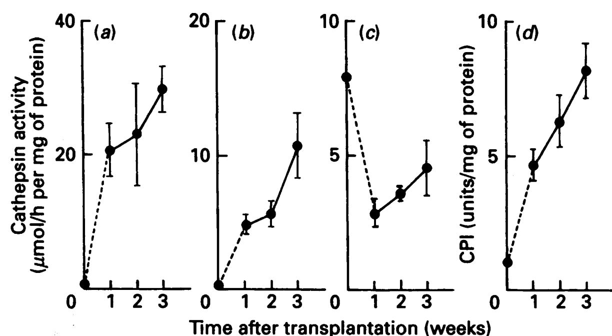

Fig. 1. Activities of cathepsins B plus L (*a*), B (*b*) and H (*c*), and amount of CPI (*d*), in normal muscle xenografts 1–3 weeks after transplantation into nude mice

Muscle extracts were prepared as described in the Experimental section. Cathepsins B-plus-L, B and H activities were fluorometrically assayed with Z-Phe-Arg-MCA (pH 5.5), Z-Arg-Arg-MCA (pH 6.0), and Arg-MCA (pH 6.8) respectively [35]. The fluorescence was measured with a Hitachi MPF-4 spectrofluorimeter with excitation and emission wavelengths set at 380 and 460 nm respectively. The amount of CPI was determined by measuring inhibition of activity of papain. Papain activity was assayed as Bz-Arg-Nap hydrolysis. A unit of inhibition is defined as the amount causing a 1 unit decrease in papain activity. These results are expressed as μmol/h per mg of protein for cathepsins B-plus-L, B and H, and munits/h per mg of protein for CPI. All values are means ± S.D. for 7, 10 and 7 assays at 1, 2 and 3 weeks respectively. The data at zero time were assayed with biopsied muscles ($n = 7$).

mg from 0.54 ± 0.16 μmol/h per mg, and cathepsin B plus L increased to 20.92 ± 4.26 μmol/h per mg from 0.86 ± 0.25 μmol/h per mg. By 3 weeks after transplantation the activity of cathepsins B was 2.2 times the level at 1 week, and the activity of cathepsin B plus L was 1.5 times the level at 1 week. In the first week, cathepsin H activity decreased dramatically to 2.84 ± 0.66 μmol/h per mg from 7.96 ± 1.0 μmol/h per mg of biopsied muscle. In the second and third weeks it tended to recover, but the change was not significant. Change in the amount of CPI tended to parallel the activity changes of cathepsins B and B-plus-L. The activity of the cathepsins and the amounts of CPI in normal and DMD muscle xenografts 2 weeks after transplantation are compared in Table 1. The activities of cathepsins B, B-plus-L and D, and acid phosphatase and the amount of CPI all tended to be higher in DMD muscle xenografts than in normal xenografts. However, the activity of cathepsin H tended to be lower in the DMD xenografts. None of these differences, however, was statistically significant.

We determined the protein contents of cathepsins B, H and L in normal and DMD muscle xenografts 2 weeks after transplantation by e.l.i.s.a. (Table 2). The protein content of cathepsin B in DMD muscle xenografts was more than three times that in normal muscle. There was no significant difference between normal and DMD muscle xenografts in the protein content of either cathepsin H or L.

The localization of cathepsins B, H and L was studied immunohistochemically in normal and DMD muscle xenografts 2 weeks after transplantation. Histochemically, Acridine Orange-positive regenerating muscle fibres were mainly localized at the peripheral regions of the muscle xenografts. Necrotic fibres, which were not positively stained by Acridine Orange, were located centrally. Regenerating muscle fibres were stained basophilically; they contained some vesicular nuclei, and distinct nucleoli were revealed by haematoxylin/eosin stain. Positive staining of cathepsin B was seen in some regenerating muscle fibres of both normal and DMD xenografts. The staining in DMD xenografts was slightly stronger than that in normal xenografts (Figs. 2*a* and 2*b*, arrows). Cathepsin H was stained in regenerating muscle fibre cells of both xenografts, but is shown only in the DMD xenograft in Fig. 2(*c*) (arrows). Cathepsin L was not detected immunohistochemically in any muscle xenograft. Usually there was no immunoreaction in necrotic muscle fibres of either normal or DMD xenografts 2 weeks after transplantation with any of the three anti-cathepsin-IgG–HRP conjugates (Fig. 2*a*, arrowheads). Negative immuno-controls, in which primary anti-cathepsin antibodies are substituted for preimmune rabbit serum, produced no reactions in regenerating or necrotic muscle fibres (Fig. 2*d*, arrow).

To confirm the presence of cathepsins B, H and L in muscle xenograft tissue, extracts were examined by Western blotting 1, 2 and 3 weeks after transplantation. Fig. 3(*a*) shows the presence of cathepsins B, H and L in normal muscle xenografts 3 weeks after transplantation. The main protein bands are the 29 kDa band of cathepsins B and H and the 30.5 kDa band of cathepsin L. Other faintly evident 39 kDa proteins that immunoreacted with the three anti-cathepsins (B, H and L) sera were detected in normal muscle xenografts, probably indicating the presence of cathepsin proforms. The intensity of the 29 kDa band of cathepsin B in normal muscle xenografts was greater 2 and 3 weeks after transplantation, and the corresponding band in DMD was not only strongest at 2 weeks (Fig. 3*b*), but it was also slightly stronger than the 29 kDa band in normal xenografts at the same time. When a large homogenate sample was used for Western blotting, a component of 68 kDa was observed to react

Table 1. Activities of cathepsins B, B-plus-L, H and D, acid phosphatase and amount of CPI in normal and DMD muscle xenografts 2 weeks after transplantation

The assay conditions for cathepsins B, B-plus-L and H are described in Fig. 1. Cathepsin D activity was assayed with haemoglobin (pH 3.8) [36]. Acid phosphatase activity was assayed with *p*-nitrophenyl phosphate, pH 5.0 [37]. These results are expressed as munits/h per mg of protein. All values are means ± S.D. for *n* experiments.

Enzyme or inhibitor	Normal ($n = 10$)	DMD ($n = 8$)	DMD/normal
Cathepsin B-plus-L	23.46 ± 8.34	26.34 ± 8.94	1.1
Cathepsin B	5.64 ± 1.28	6.96 ± 2.48	1.2
Cathepsin H	3.64 ± 0.18	2.34 ± 0.84	0.7
Cathepsin D	1.26 ± 0.24	1.56 ± 0.24	1.3
Acid phosphatase	25.20 ± 2.52	29.58 ± 4.44	1.2
CPI	6.26 ± 1.05	7.05 ± 0.92	1.1

Table 2. Contents of cathepsins B, H and L in normal and DMD muscle xenografts 2 weeks after transplantation

The contents of cathepsins B, H and L were measured by e.l.i.s.a. with a microtitre plate as described in detail in the Experimental section. The values are expressed as means ± S.D. for *n* experiments.

	Content (ng/mg of protein)	
Enzyme	Normal ($n = 10$)	DMD ($n = 8$)
Cathepsin B	201.6 ± 67.5*	632.8 ± 167.6*
Cathepsin H	69.2 ± 20.6	86.8 ± 14.9
Cathepsin L	18.8 ± 9.7	15.0 ± 2.8

* $P < 0.001$.

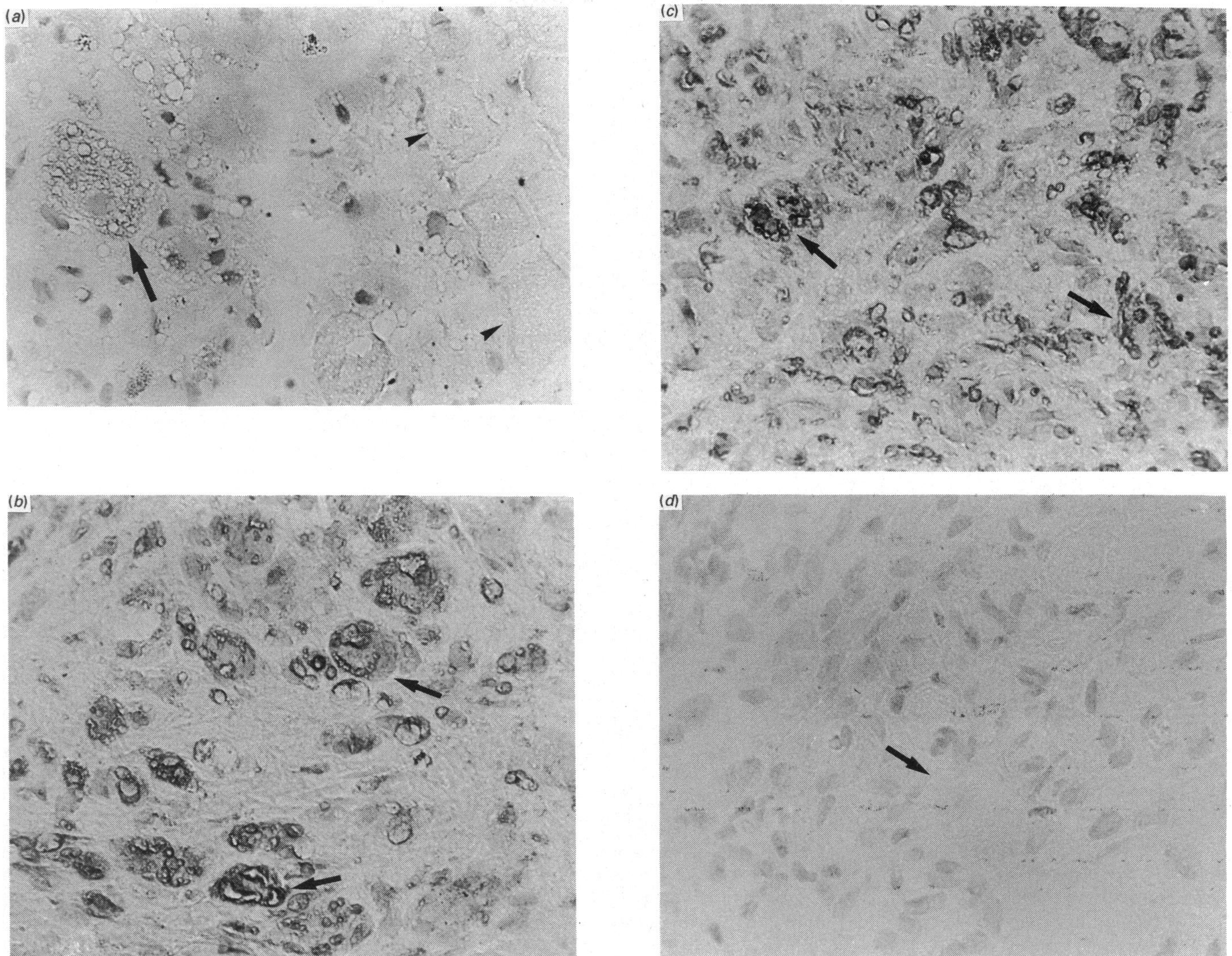

Fig. 2. Immunohistochemical localization of cathepsins B and H in normal and DMD muscle xenografts 2 weeks after transplantation

(*a*) Labelling of cathepsin B was positive in regenerating muscle fibres (arrow). No staining was observed in necrotic fibres of normal xenografts (arrowheads). Magnification × 240. (*b*) Cathepsin B was stained in regenerating muscle fibres of DMD xenografts (arrows). Magnification × 140. (*c*) Positive staining for cathepsin H was seen in the regenerating muscle fibres of DMD xenografts (arrows). Magnification × 140. (*d*) There was no immunostaining in regenerating muscle fibres in normal xenografts with preimmune rabbit serum. Magnification × 280.

with anti-(cathepsins B and H) sera. This reaction has also been observed on Western blots using high protein concentration of liver homogenates with rabbit anti-(cathepsins B, H and L) sera [42,43].

DISCUSSION

The immunodeficient nude mouse has several advantages for the study of muscle regeneration; these include no rejection of grafted tissue by the host animal, simple transplant technique and a physiological growth medium. The time courses of morphological changes of histochemically normal and DMD muscle xenografts transplanted into nude mice have been studied by Wakayama *et al.* [32,33]. Quantitative optical microscopy of transplanted xenografts of normal and DMD biopsied muscle into nude mice showed that the diameter of DMD regenerating muscle fibres was smaller 2 and 3 weeks after transplantation. These results implied excessive protein catabolism in DMD regenerating muscle fibres, so we conducted the present study of the lysosomal cysteine proteinases cathepsins B, H and L in normal and DMD muscle xenografts transplanted into nude mice.

Usually the activities of cathepsins B and B-plus-L in normal biopsied muscle were initially low and increased rapidly in the first week after transplantation. Cathepsin H activity, however, was initially relatively high in the same circumstances. It decreased sharply in normal muscle xenografts in the first week after transplantation and then tended to increase, but not significantly. There are reports that cathepsin activities derive primarily from macrophages and connective-tissue cells in many myopathies [44] and experimental myopathies [45,46]. We observed equal amounts of macrophages and connective-tissue cells in normal and DMD muscle xenografts 1 week after transplantation, as has been described previously [32,33]. The results suggest that macrophages and connective-tissue cells infiltrate into muscle xenografts, and their enzymes then contribute to the increase of cathepsins B and L and their activities. The reason for the different profile of cathepsin H activity is not yet known. A possible explanation is the presence of endogenous CPI. The amount of CPI increased each week with only slight

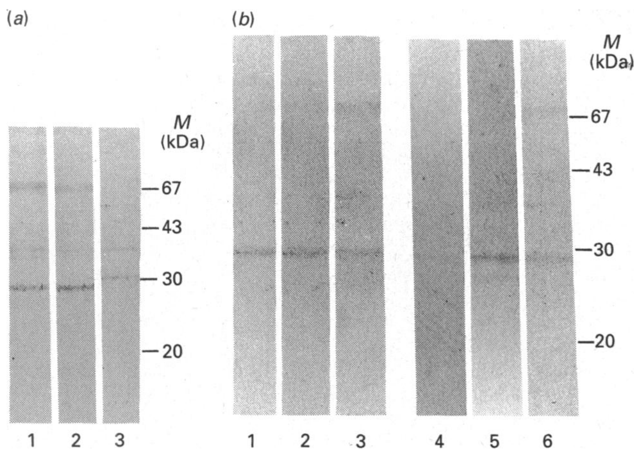

Fig. 3. Western-blotting analysis of cathepsins B, H and L

(*a*) Extracts (82 μg) of normal muscle xenografts 3 weeks after transplantation. Lanes 1, 2 and 3 were immunostained with rabbit anti-(cathepsin B, H and L) sera (1:500) respectively. (*b*) Extracts of normal (lanes 1–3) and DMD (lanes 4–6) muscle xenografts. Lanes 1 and 4, 1 week; lanes 2 and 5, 2 weeks; lanes 3 and 6, 3 weeks. Immunostaining was with rabbit anti-(cathepsin B) antiserum (1:500). Molecular masses (*M*) were taken from molecular-mass markers (Sigma) run on the same blots.

decrease in the rate in the second and third weeks. This rate of increase was in close parallel with the activity increases of cathepsins B and B plus L. Several studies have reported the existence of cystatin B, a CPI, in skeletal muscles from different sources [47–49]. Cystatin B inhibited the activity of cathepsin H about 125 times as effectively as it inhibited the activity of cathepsin B [50]. This suggests that CPI may primarily inhibit cathepsin H activity in homogenate of muscle xenografts.

The cathepsin B and B-plus-L activities were increased 2 and 3 weeks after transplantation. Immunohistochemical studies showed that cathepsin B was localized in regenerating muscle cells, but was usually undetected in necrotic muscle fibres of muscle xenografts 2 weeks after transplantation. Furthermore, cathepsin B was demonstrated by immunoblotting in xenografted muscle tissues 3 weeks after transplantation, both the mature form (29 kDa) and the proform (39 kDa) were present. The staining of the mature form to cathepsin B was more intense at 2 and 3 weeks than at 1 week after transplantation. It is thus likely that the increased cathepsin activities at these stages were derived, at least in part, from the xenografted original muscle cells themselves.

We could detect no statistically significant difference in the activity of cathepsins B, B-plus-L and H between normal and DMD muscle xenografts 2 weeks after transplantation. However, the protein content of cathepsin B in DMD muscle xenografts was about three times that in normal xenografts. The discrepancy of the results between enzyme activity and protein content can probably be explained by the presence of inactivated cathepsin B, which formed a complex with CPI in homogenate from DMD muscle xenografts. Western blotting showed a band of the mature form of cathepsin B to be the strongest among the homogenates from normal and DMD muscle xenografts 2 weeks after transplantation, because the complex dissociates into cathepsin B and CPI on SDS/PAGE. In addition, 2 weeks after transplantation, the positive immunostaining of cathepsin B in DMD regenerating muscle cells was obviously stronger than that in normal cells. Cathepsin H activity in DMD muscle xenografts 2 weeks after transplantation tended to be low in comparison with that in normal xenografts, despite the non-significant difference in protein content. Besides the presence of CPI, we cannot rule out the possibility that cathepsin H leaks from DMD muscle xenografts in the early stage of regeneration of transplants in nude mice. Sohar *et al.* [51] showed that only cathepsin H activity increased in the serum of DMD patients. We have observed the reduction of cathepsin H activity in biopsied muscles from patients with myopathies [43]. A small amount of cathepsin L was determined by e.l.i.s.a. in homogenates of normal and DMD muscle xenografts 2 weeks after transplantation, and faint bands of cathepsin L (30.5 and 39 kDa) were detected in normal xenografts 3 weeks after transplantation. However, no immunohistochemical reaction was observed with rabbit anti-cathepsin L antibody in normal or DMD muscle xenografts. Since it exists in muscle xenografts, cathepsin L may contribute to protein catabolism in muscle cells.

From the present results we infer that cathepsin B participates mainly in the regeneration of both normal and DMD muscle xenografts and, furthermore, in the DMD muscle fibre-wasting process during regeneration 2 and 3 weeks after transplantation into nude mice.

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