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Peter Würl · Helge Taubert · Axel Meye
Temuujin Dansranjavin · Ekkehard Weber
Dagmar Günther · Dieter Berger · Hannelore Schmidt
Henning Dralle · Friedrich-Wilhelm Rath

Immunohistochemical and clinical evaluation of cathepsin expression in soft tissue sarcomas

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Abstract Lysosomal proteases are known to enhance the spread of epithelial tumour cells, but little is known of the possible role of proteases in the growth of soft tissue sarcomas (STS). We investigated the expression of cathepsins D, B, S, H, L and procathepsin L in frozen sections of 34 STS from 34 patients by immunohistochemistry (IHC). Cathepsins D, B and H were relatively highly expressed in STS (77–91%). The expression rate of cathepsins S and L and of procathepsin L was lower (40–66%). Cathepsin S and L expression showed a moderate ($P = 0.078$ and $P = 0.019$) and procathepsin L a strong ($P = 0.00001$) correlation with the survival rate of STS patients. Cathepsin S expression is also correlated with the local recurrence rate ($P < 0.01$). Lysosomal proteases may play a role in STS progression, and cathepsin expression may also have significance as a prognostic factor in STS.

Key words Cathepsins · Immunohistochemistry · Human soft tissue sarcomas · Prognosis

Introduction

Soft tissue sarcomas (STS) are malignant mesenchymal neoplasms with an incidence around 1% among all human malignancies [8]. They grow in such a way as to give the appearance of a pseudocapsule, composed of an inner compression zone and an outer reactive zone with the extensions in the form of fingers. Through these fingers the tumour can extend and give rise to satellite lesions several centimetres away from the primary tumour [12]. The major clinical problems in the treatment of STS are the propensity of the tumour to recur locally and the fact that many patients without obvious clinical metastases are harbouring occult micro-metastases that become clinically evident later. Lymph node metastases are rare in STS [5, 9]. Despite adequate local control of the primary tumour, about 50% of sarcoma patients will succumb to distant metastatic disease [24].

Cathepsins are lysosomal proteinases which account for about 70–80% of the proteolytic activity of the lysosomes. Cysteine proteinases (such as B, H, S, L), together with the aspartyl protease cathepsin D, are thought to be the major proteinases involved in intracellular protein degradation [16, 17, 18]. They also play a part in antigen presentation [13, 29]. In vitro examinations have shown that cathepsins can be involved in invasive tumour growth, both indirectly, by activating other enzymes, and directly, by decomposing extracellular matrix constituents [2, 6, 16, 26, 34]. Furthermore, transformed, in contrast to normal cell lines, may show a greatly increased expression of cathepsins L, D, B. The degree of this increase correlates directly with the metastatic potential of these cells [4, 34, 35]. The role of cathepsins during benign or malignant growth processes has been investigated in carcinomas, fibroblast cell lines [34, 35], connective tissues [11, 39], melanomas [10, 15] and gliomas [23, 27, 33], but not, however, in STS.

Our aim was to characterize the expression of cathepsins D, B, S, H, and L and of procathepsin L in STS immunohistochemically and to compare the results with histomorphological and clinical variables.

Materials and methods

The tumour material originated from 34 STS patients (17 female and 17 male) with an average age of 57 (21–85) years. These patients had operations for STS primary tumours ($n = 20$; 59%) or

P. Würl · H. Dralle
Clinic of General Surgery,
Martin-Luther-University of Halle-Wittenberg, Germany

H. Taubert (✉) · A. Meye · T. Dansranjavin · D. Berger
H. Schmidt · F.-W. Rath
Institute of Pathology,
Martin-Luther-University of Halle-Wittenberg,
Magdeburger Strasse 14, D-06097 Halle/S., Germany
Tel.: (49) 345-557-1293, Fax: (49) 345-557-1295
e-mail: mgopu@mlucom2.urz.uni-halle.de

E. Weber · D. Günther
Institute of Physiological Chemistry,
Martin-Luther-University of Halle-Wittenberg, Germany

recurrences (n = 14; 41%) in the Clinic of General Surgery, University of Halle. The frozen tumour material was prepared from 9 malignant fibrous histiocytomas (MFHs), 7 liposarcomas (6 myxoid and 1 inflammatory liposarcomas), 5 fibrosarcomas, 6 malignant neural tumours (MNT, 6 malignant schwannomas), 3 leiomyosarcomas, 2 rhabdomyosarcomas and 2 synovial sarcomas. The localizations were: in 21 (61%) cases, extremities, in 7 (21%), retroperitoneal, in 4 (12%), the chest wall, in 2 (6%) cases, the head. Two of the tumour samples were grade 1, 21 were grade 2, and 11 were grade 3 according to the grading system of van Unnik [41].

For evaluation we only included patients whose primary tumour had been removed as an R0-resection. Of the 34 patients, 16 (47%) died of the tumour an average of 24 months (10–62 months) after primary resection, whereas 18 (53%) patients are alive (average observation period 41 months; 15–90 months). Of the 34 patients, 15 developed recurrences. During the subsequent course of disease, 16 of the patients developed distant metastases and 3 lymph node metastases. Staging of the primary tumours of the patients yielded 4 stage I, 16 stage II, 11 stage III, 3 stage IV.

For verification of cathepsin D, the rabbit polyclonal antibody A561 (Dako, Hamburg, Germany) and the mouse monoclonal antibody Ab-1 (Oncogene Science, Manhasset, N.Y.) were used. For cathepsin L, a sheep polyclonal antibody (no. 06–0008; BioAss, Gießen, Germany) was applied (dilution 1/1000 in each case). Additionally, we used the following noncommercial antibodies (Dr. H. Kirschke, Halle and Dr. B. Wiederanders, Jena): for verification of cathepsin L, a goat polyclonal antibody (17.1 ng/ml), for procathepsin L a mouse monoclonal antibody (6.0 ng/ml), for cathepsin B a rat polyclonal antibody (11.9 ng/ml), for cathepsin H two rabbit polyclonal antibodies (14.8 ng/ml) and for cathepsin S a rabbit polyclonal antibody (9.1 ng/ml) [20, 43, 44]. All noncommercial antibodies were diluted 1/1000. All but one of the antibodies (one cathepsin H antibody against rat) were raised against human cathepsins.

Frozen 6-µm sections from all samples were placed on poly-L-lysine coated slides and fixed in ice-cold acetone for 20 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 95% methanol at 4°C for 20 min. Nonspecific serum-binding sites were blocked with 20% normal serum of the carrier protein solution (sheep, rabbit, mouse, goat) diluted in phosphate-buffered saline (PBS) at pH 7.6 for 30 min. Incubation with the primary antibody at a concentration mentioned above was performed at 4°C for 24 h. Incubation was followed by a washing step (PBS, pH 7.4) for 5 min and detection of the primary antibody by an LSAB-Kit (Dako). Nuclear counterstaining was performed with Mayer's haemalaun.

Normal macrophages served as an internal positive control for all cathepsins. As an external positive control, specimens from breast cancer (cathepsin D), spleen (cathepsin S) and kidney (cathepsin B) were used. Negative control was achieved by omission of the primary antibody. The material was evaluated by two independent assessors. The slides were considered positive if more than 10% of the tumour cells showed staining.

Survival curves were plotted using the method of Kaplan and Meier, and significance for overall survival was calculated using the log-rank test. Relationships between cathepsin expression and relapse rate were examined using contingency tables and the Chi-square test.

Table 1 Immunohistochemical detection of cathepsins D, B, H S, L and procathepsin L in STS according to the patients (primary tumour or recurrence; *PT* primary tumour, *R* recurrence, *pos* positive, *neg* negative)

Expression of cathepsins	(n=34) pos/neg	positive (%)	PT (n=20) pos/neg	R (n=14) pos/neg
D[a]	26/8	76	13/7	13/1
D[b]	28/6	82	14/6	14/0
B	30/4	88	17/3	13/1
H	31/3	91	17/3	14/0
H[b]	33/1	97	19/1	14/0
S	22/12	65	9/11	13/1
Pro-L	19/15	56	11/9	8/6
L	14/20	41	7/13	7/7

[a] human cathepsin D antibody (Dako)
[b] human cathepsin D antibody (Oncogene Science)
[c] rat cathepsin H antibody [44]

Results

Within each of the tumour samples, at least two cathepsins could be identified immunohistochemically. Expression was independent of the entity, localization or size of the primary tumour. Samples with a positive IHC expression originated mostly from patients whose primary tumour already showed a higher grade of malignancy (Table 1).

The cathepsins examined can be categorized into one group consisting of cathepsins D, B, and H, with a very high rate (77–91%) of expression, and a second group consisting of cathepsins S, L, and procathepsin L, with a medium rate (40–66%) (Table 1). Thus, only for cathepsins S and L and procathepsin L is it useful to compare clinical data and expression rates (Table 2). Patients with cathepsin S-positive tumours had a poorer prognosis than patients with negative immunohistological findings (P = 0.078; Fig. 1). For cathepsin L, this connection is even more distinct (P = 0.019; Fig. 2). However, the clearest prognostic relevance of an immunohistochemical verification is shown for procathepsin L (P = 0.00001; Fig. 3).

Out of 14 patients who developed a local recurrence within the observation period, 13 tested positive for cathepsin S (93%) (Fig. 4). In the case of recurrence-free patients (n = 20), only 9 patients (45%) showed cathepsin S positivity (P < 0.01). However, such a relationship was not statistically verifiable for cathepsin L and procathepsin L (Table 2).

Table 2 Correlation of cathepsins S, L and procathepsin L expression with clinical data of primary tumours of the STS patients (*d*-dead, *a* alive, *IHC* immunohistochemistry, *pos* positive, *neg* negative

Cathepsin IHC	n (%)	Survival d/a	Grading PT 1/2/3	Staging PT I/II/III/IV	Relapse Yes/No	M_1/M_0
S pos	22 (65)	14/8	2/11/9	2/10/8/2	13/9	13/9
S neg	12 (35)	2/10	2/6/4	2/6/3/1	1/11	3/9
Pro-L pos	20 (59)	16/4	3/7/11	3/7/8/3	9/11	15/5
Pro-L neg	14 (41)	0/14	1/9/3	1/9/3/0	5/9	1/13
L pos	14 (41)	11/3	1/5/8	1/5/6/2	7/7	9/5
L neg	20 (59)	5/15	3/11/6	3/11/5/1	7/13	7/13

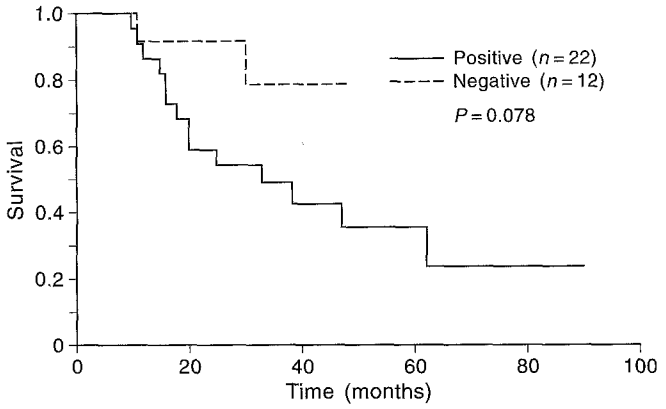

Fig. 1 Kaplan-Meier curve for immunohistochemical cathepsin S expression

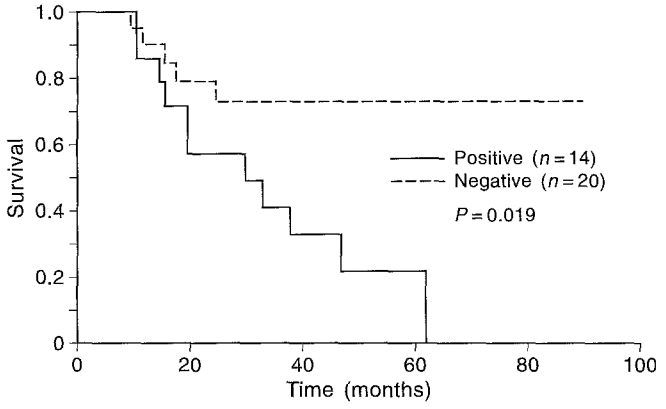

Fig. 2 Kaplan-Meier curve for immunohistochemical cathepsin L expression

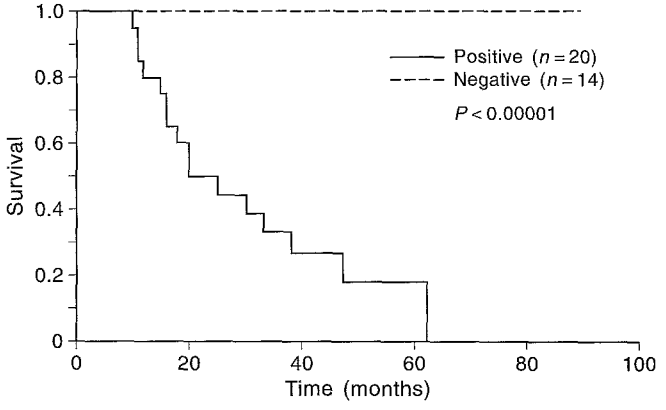

Fig. 3 Kaplan–Meier curve for immunohistochemical procathepsin L expression

Discussion

In this, the first study to report the presence and localization of cathepsins in STS, we have determined the presence of cathepsins D, B, H, S, and L, and of procathepsin L in STS using an immunohistochemical approach. Cathepsins D, B and H showed positive staining in about 80% of cases (Table 1). Argument about these very high

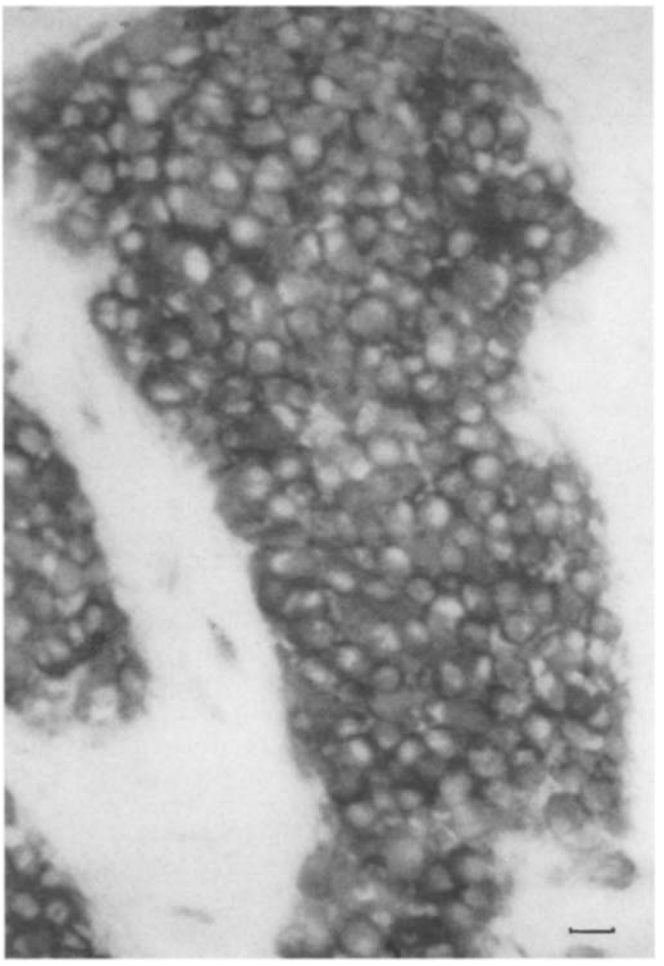

Fig. 4 Immunohistochemical staining of a synovial sarcoma with cathepsin S antibody. ×800, *bar* 10 μm)

expression rates is difficult, because comparable results exist only for carcinoma. Sarcomas differ from carcinomas in their mode of invasion, which is evident in their very low lymph node metastasis rate and in the formation of a pseudocapsule and of skip metastases [5, 8, 9, 24, 41]. It is possible that cathepsins have a substantial influence on the local infiltration mechanism, with tumour spreading in a pattern reminiscent of fingers and skip metastases, which are related to high expression rates for cathepsin D, B, and H.

For monoclonal and polyclonal antibodies to cathepsins B, D, H, IHC detection rates between 70% and 100% have been reported for breast cancer, prostatic carcinoma, melanoma and glioblastoma [3, 10, 15, 23, 32, 33]. For laryngeal and bladder cancer (using monoclonal antibodies) and for anal carcinoma, (using polyclonal antibodies to cathepsin D) detection rates around 50% or less were found [7, 14, 28]. Owing to their high expression rate, cathepsins D, B, H did not show any useful correlations with clinical variables in our studies. However, it is known that for a number of carcinomas (e.g. like anal, prostatic, and breast carcinomas) a relationship between immunohistochemical detection rate and prog-

nosis has been found. Correlations with such variables as survival time, hormonal receptor status and recurrence rate were found [7, 14, 22, 28, 32, 37, 38, 42]. However, other studies in breast cancer patients have not shown a correlation between expression of cathepsins D, B, and L and the clinical course [1, 3]. For cathepsin B in general, as for cathepsin D, a high correlation with an unfavourable clinical course and a poor prognosis was found. For prostatic carcinomas, a clear correlation was determined between expression rate, extracellular matrix degradation, neoangiogenesis, and tumour progression or prognosis [32]. Similar results were found by other authors for malignant gliomas [23, 27, 33], malignant melanomas [10, 15], and bronchial carcinomas [37]. There is only a small number of immunohistochemical studies on cathepsin H expression in human malignomas, but each of them has confirmed a positive expression [15, 40].

In contrast to this, cathepsins S and L and procathepsin L showed a moderate expression rate (40–61%), allowing correlation with clinical data. Our results point towards a correlation between immunohistochemical cathepsin S verification and overall survival time (Fig. 1). There is a significant correlation between cathepsin S positivity and local recurrence rate (Table 2), and cathepsin S expression may thus influence the high local recurrence rate without affecting systemic tumour spread. This suggestion implies the possibility of a prognostic value for cathepsin S positivity in our patients.

For cathepsin L we found a clear, and for procathepsin L a highly significant, correlation between immunohistochemical positivity and overall survival in STS (Figs. 2, 3). Correlation with the local recurrence rate is not apparent (Table 2). Thus, for cathepsin S, correlation with local tumour events is more probable, and for cathepsin L and procathepsin L a correlation with systemic dissemination and thus with overall prognosis is more likely.

Within this framework it is interesting that cathepsin S has been reported to play a significant part in the development and progression of degenerative diseases of the brain, but not in the progression of malignant tumours [21].

In malignant mesenchymal cell lines an increase in cathepsin L or procathepsin L expression was found, depending on malignancy grade [4, 31, 36]. This correlates with the results we achieved with solid mesenchymal tumour material. Similar results have been found for malignant melanoma [15, 25, 30]. The correlation between the expression rate of cathepsins S, L, procathepsin L and survival suggests that their expression affects the prognosis at least in STS. It is possible that they, like the highly expressed cathepsins D, B, and H, are able to influence tumour behaviour through their ability to lyse matrix proteins [2, 6, 11, 19, 21].

These data suggest that the study of cathepsin expression in a larger number of patients will help us to evaluate the role of cathepsins in predicting the clinical course and prognosis of STS patients.

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