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Hyaluronidase in sera of tumour-bearing nude mice

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Cancer cell lines often secrete hyaluronidase, suggesting that this enzyme could be used as a marker of growing tumours. We have measured hyaluronidase in the sera of non-grafted mice and mice grafted with human tumour-derived hyaluronidase-secreting H460M and SA87 cells or non-secreting CB 193 cells. Mouse serum hyaluronidase was measured at pH 3.8 using the enzyme-linked sorbent assay (ELSA) technique by reference to human serum whose activity at pH 3.8 was determined by the Reissig technique. The serum hyaluronidase in non-grafted mice ranged from 310–520 mU l^{-1} (mean $\pm$SD 432 $\pm$70 mU l^{-1}, median 440 mU l^{-1}). Hyaluronidase increased in the sera of tumour-bearing mice grafted with H460M cells or with SA87 cells, but not in the sera of mice grafted with CB 193 cells. Serum hyaluronidase activity in H460M or SA87 tumour-bearing mice correlated with the tumour mass, increased with time, and decreased after tumour removal. Zymography detected two different hyaluronidase forms in the sera of non-grafted mice: type 1 had only one hyaluronidase band and type 2 had five different bands. In both types, enzyme augmentation in tumour-bearing mice correlated with the presence of an additional enzyme band that was not seen in normal sera and that migrated as the cancer cell enzyme did; there was no augmentation of the normal isoform(s). These results show that serum hyaluronidase can be used to follow the development of tumours in mice grafted with hyaluronidase-secreting cells.

Introduction

An important tool for following growing tumours, which are not otherwise measurable when they develop inside the body, would be a serum marker relevant to cancer cells. Several human cancer-derived cell lines release hyaluronidase in culture medium (Victor *et al.* 1997), suggesting that this enzyme could be assayed in sera from tumour-bearing mouse as a marker to follow tumour growth. However, fibroblasts and monocytes participate in tumour stroma formation and also produce hyaluronidase (Stair-Nawy *et al.* 1999, Girard *et al.* 2002), raising the question of whether cancer-associated hyaluronidase originates from cancer cells or from the stroma. In this study we have measured hyaluronidase in the sera of mice grafted with hyaluronidase-secreting and non-secreting human cancer-derived cells. Enzyme activity was followed after grafting and tumour removal and the enzyme was characterized using zymography.

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Materials and methods

Mice

Rj:NMRI-nu (nu/nu) pathogen-free, 6 week old female mice were purchased from Centre d'élevage Janvier (Le Genest s/Isle, France). Ico:SWISS-nu/nu 6 week old female mice were purchased from Iffa Credo (Saint Germain sur l'Arbresle, France). Similar results were obtained with both lines in grafting.

Cell lines

NCI-H460 (a human lung cancer-derived cell line) cells were obtained from the American Tissue Culture Collection. The H460M cell line was derived from metastases of NCI-H460 cells in nude mouse lung (Corti *et al.* 1996). The SA 87 cell line was developed by R. Maunoury from a gastric cancer brain metastasis (Victor *et al.* 1997). All three cell lines released hyaluronidase in the culture medium. The glioma-derived cell line CB 193 was obtained in our laboratory and did not release hyaluronidase in the culture medium.

Cell grafting and tumours

Cells were cultivated without serum for 24 h, and 5 million cells in 200 µl RPMI 1640 medium were subcutaneously injected into nude mice flanks. After cell grafting, blood was taken weekly from the eye vein under general anaesthesia for serum hyaluronidase determination.

The grafted mice were followed up for as long as the size of the tumour allowed. Tumours were removed surgically under general anaesthesia and weighed. Blood was taken from the eye vein before tumour removal.

Blood samples were also collected from 15 non-grafted mice.

Hyaluronidase serum assay

The hyaluronidase activity in the mice sera was measured using the ELSA technique with reference to a human serum sample.

A human serum sample was assayed for hyaluronidase using the method of Reissig (Reissig *et al.* 1955, Asteriou *et al.* 2001). One enzyme unit corresponds to 1 µM of reducing *N*-acetylglucosamine ($M_r = 221.2$) released from hyaluronan per minute. This serum was used as the reference to assay the hyaluronidase activity of the culture media using the ELSA technique as previously described (Delpech *et al.* 1995). The use of human serum as a reference to assay mouse serum hyaluronidase was possible since mouse and human serum hyaluronidase activities have similar characteristics: they have the same optimum pH activity; the salt and protein concentrations must be adjusted to obtain optimum enzyme activity (Maingonnat *et al.* 1999); digestion is optimum at 37°C and is abolished by preheating the serum at 60°C; and serum activity is not suppressed after incubation and can still digest substrate. Moreover, the Reissig technique requires a large amount of serum (1 ml), which cannot be obtained from a mouse, whereas the ELSA technique allows the measurement of activity in very small volumes of serum (a few microlitres). The technique was performed as follows. Briefly, plastic microtest plates (Nunc Maxisorp F96) were coated with 0.1 ml of 100 mg l^{-1} hyaluronan in 0.1 M sodium bicarbonate overnight at 4°C, followed by incubation with a solution containing 1 g l^{-1} bovine serum albumin (BSA) in 0.1 M Tris HCl buffer, pH 7.4, at 37°C in a wet chamber for 30 min. Hyaluronidase destroys the capacity of the plates to bind alkaline phosphatase-linked hyaluronectin (AP-HN), leading to lowering of the alkaline phosphatase activity. The mouse serum samples were diluted in citrate/NaCl buffer (0.1 M citric acid containing 0.05 M NaCl supplemented with 50 µg BSA ml^{-1} adjusted at pH 3.8 with sodium hydroxide), and 0.1 ml from the diluted samples was incubated in wells for 2 h at 37°C. Plates were rinsed with water, with 0.1 M acetic acid and again with water to remove any protein bound to hyaluronan. Wells were incubated with AP-HN diluted in 0.1 M Tris HCl buffer containing 0.5 M NaCl, 1 g l^{-1} BSA, 0.25 g l^{-1} sodium azide and 0.5 g l^{-1} Tween 20, pH 7.4, for 3 h at 4°C. Alkaline phosphatase activity was detected with *p*-nitrophenyl phosphate by measuring the absorbance at 405 nm. The hyaluronidase standards were from 40 to 1.2 mU l^{-1}, corresponding to human serum dilutions from 1:1000 to 1:32 000. Mouse sera diluted to 1:10 and 1:100 gave values belonging to the linear part of the standard curve. Assays were performed in duplicate. The intra-assay and inter-assay variations were lower than 5%. The limit of detection of hyaluronidase activity corresponded to a decrease in alkaline phosphatase activity of 20% of the maximum activity found with the control without enzyme. For the conditions used (i.e. incubating the enzyme samples for 2 h), the detection threshold was 0.6 mU l^{-1} (a 1:64 000 dilution of the reference normal human serum). The results were not altered by the addition of hyaluronan-binding protein in the reaction medium but might be lowered by the presence of high concentrations above 1 mg l^{-1} of the substrates (i.e. hyaluronan or chondroitin) in the serum samples, but this is not the case with mouse sera. Results were recorded with a Powerwave device (Biotek Instruments, Winooski, Vermont) and computer calculations were performed online using the KC4 data reduction package.

Correlation studies were done using the Pearson correlation test.

Zymography of serum hyaluronidase

Zymography was used to characterize the hyaluronidase molecular mass in the mice sera as described elsewhere (Fiszer-Szafarz 1984, Bertrand *et al*. 1997). Briefly, electrophoresis was run on 8.5% polyacrylamide gel with or without 0.1% sodium dodecyl sulphate (SDS) and 20 μg ml^{-1} hyaluronan. Omitting SDS in the gel facilitates the characterization of human-type hyaluronidase in mouse sera. When SDS was used, the gel was submitted to three additional washings: twice for 30 min in 5% Triton X-100 in water, and once for 30 min in 0.05% Triton X-100. Gels were then washed twice for 30 min in hyaluronidase buffer containing 1 g l^{-1} BSA. The gel was incubated at 37°C overnight in the hyaluronidase buffer. After two 30 min washings in water, the gel was incubated in 1 mg ml^{-1} protease from *Streptomyces griseus* (Sigma, 4.6 units mg^{-1}) in phosphate buffered saline for 8 h at 37°C, washed three times in water, and placed in 50% formamide in water overnight at 4°C. The gel was placed in Stains-all (0.05 mg ml^{-1} in 50% formamide in water), which stains glycosaminoglycans blue. Translucid colourless areas in the gel indicate hyaluronan digestion.

Results and discussion

Hyaluronidase serum assay

The mean ± SD hyaluronidase activity in the sera from 15 non-grafted mice, estimated using the ELSA technique with reference to a human serum sample, was 432 ± 70 mU l^{-1}. The limit values were 310 and 520 mU l^{-1}, and the median was 440 mU l^{-1}. The mean hyaluronidase activity in eight H460M tumour extracts was 92 ± 36 mU g^{-1}.

Activity was increased in the sera of mice grafted with hyaluronidase-secreting cells. The increase in serum activity correlated with tumour weight in 19 H460M tumour-bearing NMRI nude mice ($p = 4.10^{-6}$) and in eight SA87 tumour-bearing Swiss nu/nu mice ($p = 3.10^{-4}$) (Figure 1). In both series, a significant increase ($>$ mean ± 2 SD) in individual mouse serum hyaluronidase activity was detected when the tumour weight reached 0.5 g.

The serum hyaluronidase activity decreased when the primary tumours were removed in 11 out of 11 grafted mice, and increased again in eight out of 11 cases at primary tumour recurrence; Figure 2 shows two typical cases. In contrast, no increase of serum hyaluronidase was recorded in mice grafted with CB 193 cells throughout the experiment, even 270 days after grafting, although some tumours weighed more than 10 g.

Zymography of serum hyaluronidase

In a series of 15 non-grafted mice, two different phenotypes were observed, one with only one major band (type 1), the other with five bands detected in the gel (type 2). For both types, the augmentation of serum hyaluronidase correlated with the secretion in the mouse sera of a new hyaluronidase band (Figure 3) without any augmentation of the one or several normal hyaluronidase isoforms. When electrophoresis was run without SDS, the new band was much slower but more distinctly separated than in the SDS gel. Using the SDS gel electrophoresis method, the observed molecular mass of the new enzyme was 66 kDa, similar to that found in the cell culture medium of the H460M cell line (Delpech *et al*. 2001) or the SA87 cell line (Delpech *et al*. 2002). The intensity of the new hyaluronidase

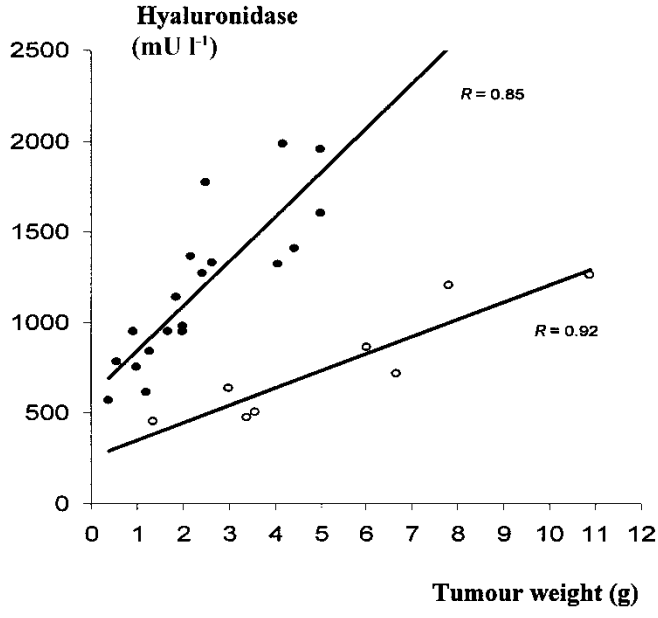

Figure 1. Serum hyaluronidase activity in 19 H460M (filled circles) and eight SA87 (open circles) tumour-bearing nude mice. Hyaluronidase concentrations correlated with the tumour weights.

band increased after grafting, which correlated with the increase in hyaluronidase activity assayed in sera.

These results showed that the hyaluronidase found in the cell culture media of the H460M and SA87 cell lines was present *in vivo* in the sera of mice grafted with hyaluronidase-secreting H460M or SA 87 cells and was not seen in sera of mice

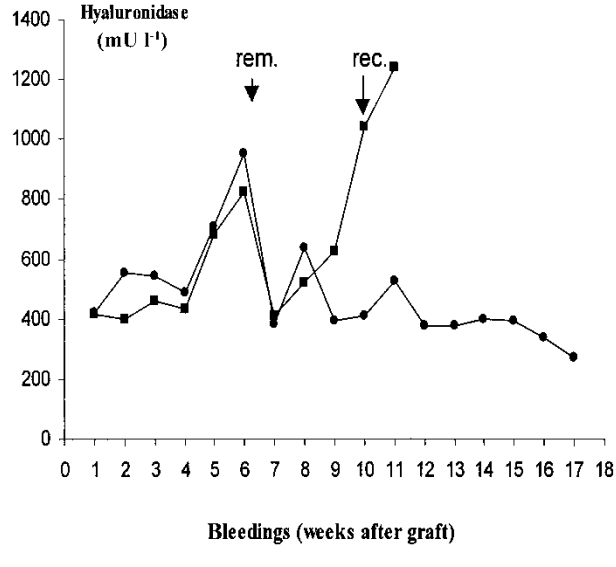

Figure 2. Serum hyaluronidase levels in two examples of H460M tumour-bearing mice. In one mouse, the hyaluronidase activity returned to normal values after tumour removal (rem.). In the other mouse the serum hyaluronidase increased with tumour recurrence (rec.).

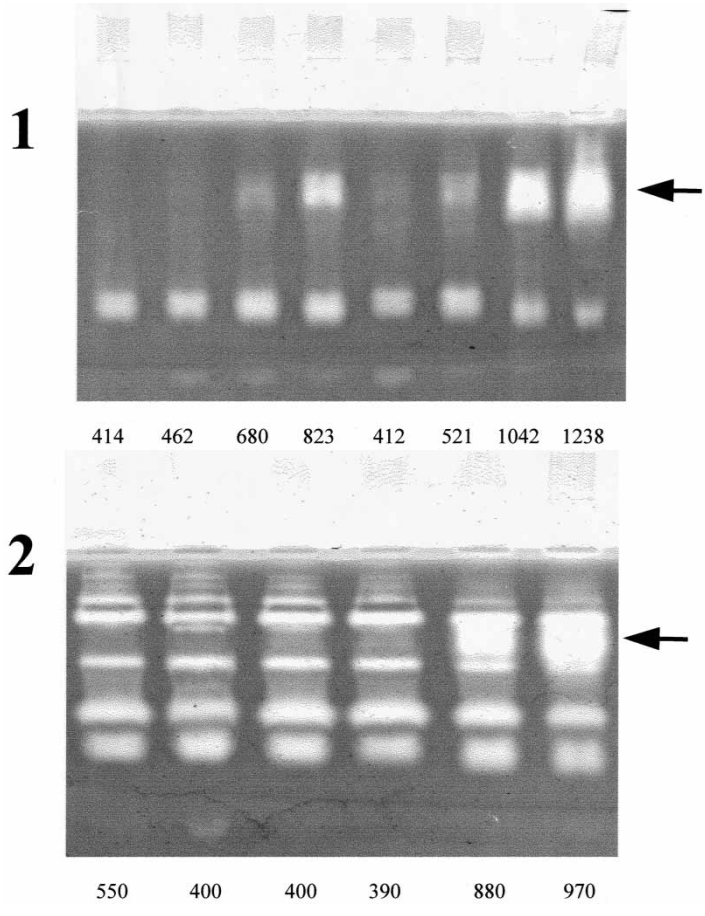

Figure 3. Zymogramm of serum hyaluronidase in the sera of two nude mice with different hyaluronidase phenotypes, grafted with hyaluronidase-secreting H460M cells. The numbers correspond to hyaluronidase concentrations in mU l^{-1}, measured every week starting from the grafting day. A new band (arrows) indicates H460M hyaluronidase accumulation in the sera. In panel 1, the intensity and concentration decreased at the fifth bleeding, following tumour removal, and augmentation occurred with tumour recurrence.

grafted with non-hyaluronidase-secreting CB 193 cells. The serum hyaluronidase activity increased with tumour size, and a new enzyme form was seen with a molecular mass similar to that of the human cancer cell hyaluronidase and different from that of the mouse serum hyaluronidase isoforms.

Since mouse serum hyaluronidase remains at a constant level during the growth of tumours derived from non-hyaluronidase secreting cells, and that normal isoforms do not increase even when hyaluronidase-secreting tumours are growing, we may conclude that the increase in serum hyaluronidase is due to secretion by the cancer cells and does not originate from the stroma. When cancer cells of human origin secrete hyaluronidase, the human-type enzyme can thus be used as a tumour growth marker. In the mouse, this can be useful in following the growth of grafted human cancer cells when they are growing in sites where tumours are not detectable or not measurable (e.g. intraperitoneal grafts), or to detect the

development of internal metastases after removal of the primary tumour. In the human, an assay for measuring cancer cell hyaluronidase activity would need a specific reagent to discriminate between the cancer cell enzyme and normal hyaluronidase(s) present in serum. It seems likely that antibodies are the best tool for this. Nevertheless, the interest of the measurement would be based on variations in enzyme concentrations in sera, as shown here in an experimental model, rather than on the expectation of a cancer-specific molecule.

Elevated hyaluronidase in tumours produces hyaluronan-derived oligosaccharides, whose angiogenesis-promoting activity has been clearly established (West and Kumar 1989, Slevin *et al*. 2002). Hyaluronidase follow-up in sera could be a valuable marker in monitoring cancer development.

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