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Extracellular protease mRNAs are predominantly expressed in the stromal areas of microdissected mouse breast carcinomas

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Solid tumors synthesize a number of extracellular matrix-degrading proteases that are important for tumor progression. Based on qualitative *in situ* hybridization studies in human cancer tissue, a range of components involved in proteolysis appear to be expressed by stromal cells rather than cancer cells. We have now used laser capture microdissection and real-time PCR to quantify the mRNA expression of components of matrix-degrading proteolytic systems in cancer and stromal areas of mouse mammary tumors genetically induced by the polyoma virus middle T (PyMT) antigen. We examined the mRNA levels of urokinase plasminogen activator, plasminogen activator inhibitor 1 and the matrix metalloproteases MMP-2, -3, -11, -13 and -14, and found that all these seven genes are predominantly expressed by stromal cells. Our results were qualitatively supported by *in situ* hybridization analysis of the expression of mRNAs for MMP-2, -3 and -13 in the PyMT tumors. Statistical analyses indicated that the quantitative expression patterns observed in cancer and stromal cells isolated from individual tumors from different PyMT mice are quite reproducible. The methodology described in this study provides excellent tools to study the possible interactions between cancer and stromal cells during the development of breast cancer, and the results suggest that stromal cells are involved in carcinogenesis and tumor progression, which may have important implications for the biology and therapy of cancer.

Introduction

Although cancers typically have a much less higher-order structure than the tissue from which they arise, they are still complex, structured assemblies of the malignant epithelial cells and several stromal cell types. Interestingly, cancer cells appear to recruit neighboring stromal cells to synthesize components that are important for cancer growth, invasion and metastasis (reviewed in refs 1–4). Proteolytic degradation of the extracellular matrix is critical during invasion of cancer. The serine protease plasmin and several matrix metalloproteases (MMPs) are all involved in tumor extracellular proteolysis, which is regulated by a number of functionally different components, i.e. proteases, their receptors, activators and inhibitors (5–8). Plasmin is formed by cleavage of the inactive zymogen plasminogen (Plg) by specific plasminogen activators (PAs) of the tissue type or urokinase type (uPA). The specific uPA receptor (uPAR) stimulates the uPA-catalyzed plasmin generation and localizes it to cell surfaces. PA inhibitors, such as PA inhibitor type 1 and 2 (PAI-1 and -2), specifically inhibit the PAs (5,6,9). MMPs are also activated from pro-enzyme forms and their activity is blocked by specific tissue inhibitor of metalloproteases (7,8).

In situ hybridization (ISH) and immunohistochemical studies indicate that components of matrix-degrading proteolytic systems are often synthesized by stromal cells and not by the cancer cells in many human cancers. For example, in human ductal breast carcinomas, uPA and PAI-1 are synthesized primarily by myofibroblasts surrounding the cancer cells (10,11). Similarly, MMP-2, -3, -9, -11, -13 and -14 are synthesized either by stromal fibroblasts, infiltrating macrophages or vascular pericytes in ductal breast carcinomas (12–17).

We have previously studied the expression and function of uPA in a mouse model of breast cancer genetically induced by the polyoma virus middle T antigen under transcriptional control of the mouse mammary tumor virus long terminal repeat (PyMT mice). Female FVB-PyMT mice develop primary mammary tumors at the age of 6–7 weeks and after an additional 7 weeks, lung metastases are observed in nearly 100% of the mice (18,19). We found that uPA in the primary PyMT tumors are predominantly expressed by stromal cells, similar to the expression pattern in human ductal carcinomas, using ISH and immunohistochemistry. Development and growth of primary tumors in PyMT mice is not affected by deficiency in the genes for Plg or uPA, whereas lung and lymph node metastasis is significantly reduced in PyMT mice lacking either of these genes (19,20). This indicates a role of plasminogen activation catalyzed by stromal cell-derived uPA in metastasis in this cancer model.

The advent of microdissection techniques, such as laser capture microdissection (LCM), has made it possible to isolate distinct cells from tissue sections (21,22). This enables the isolation of populations of cancer and stromal cells from individual tumors and use of the isolated cell samples for subsequent gene expression analyses. Quantitative real-time PCR (qPCR) is an excellent tool for quantitation of the small amounts of RNA from such microdissected cells. Several qPCR analyses can be performed on one microdissected sample without previous amplification of the RNA. This allows the quantitative determination of several mRNAs expressed by a very small and specific population of cells.

Abbreviations: C_T, cycle threshold; ISH, *in situ* hybridization; LCM, laser capture microdissection; MMP, matrix metalloprotease; PA, plasminogen activator; PAI, PA inhibitor; PyMT, polyoma virus middle T; qPCR, quantitative real-time PCR; uPA, urokinase type PA; uPAR, uPA receptor.

We have now used LCM to isolate populations of cancer and stromal cells, respectively, from 10 primary mammary tumors from five PyMT mice, and qPCR analysis to quantitate mRNA levels for uPA, PAI-1, MMP-2, -3, -11, -13 and -14 in the cell samples. We also studied the expression of mRNAs for MMP-2, -3 and -13 in primary PyMT tumors by ISH for an independent qualitative confirmation of the results.

Materials and methods

FVB-PyMT mice

Heterozygous FVB/N-TgN(*MMTVPyVT*)634Mul females (referred to as FVB-PyMT) (18) were genotyped as described (20,23). All transgenic mice from the resultant cohort were killed at the age of 13–15 weeks, at which point they all had several mammary tumors. Mice for which tissue was to be used for LCM were anesthetized by intraperitoneal administration of a 1:1 mixture of Dormicum (Roche A/S, Basel, Switzerland) and Hypnorm (Janssen-Cilag Ltd, High Wycombe, UK) and killed by intracardial perfusion with 10 ml ice-cold phosphate-buffered saline (PBS). From each mouse, a minimum of two mammary glands were excised, embedded in OCT compound and snapfrozen in isopentane cooled by dry ice. In the present study, we included only primary tumors from mice that had visible surface metastases of the lung. Mice for which tissue was to be used for ISH were anesthetized by intraperitoneal administration of a 1:1 mixture of Dormicum and Hypnorm, and perfused intracardially with 10 ml ice-cold PBS followed by 10 ml 4% paraformaldehyde in PBS. Mammary glands were then excised and processed into paraffin as described previously (23).

LCM

The isolation of cells from histological sections by LCM was performed essentially as described (21,22,24). Cryostat sections of 6 μm were prepared, and the sections were kept on dry ice, or at −80°C, until they were subjected to LCM. Just before the procedure, the sections were fixed in 70% ethanol for 10 s and stained with hematoxylin and eosin by immersion using the following protocol: 10 s deionized H_2O, 10 s hematoxylin, 2 × 10 s deionized H_2O, 10 s 70% ethanol, 10 s eosin Y (alcoholic), 2 × 10 s 95% ethanol, 2 × 10 s 100% ethanol, 30 s xylene. LCM was performed using an Arcturus PixCell II apparatus, with a 7.5 μm laser beam, power settings of 35–45 mW, and a laser pulse duration of 0.85 μs. Cancer and stromal cells were identified by their morphological appearance. Both cancer and stromal cells were primarily isolated from tumor areas with well-defined cancer cell islands. Cancer cells and stromal cells were isolated from adjacent tissue sections. In the majority of the cases, cancer cells and stromal cells were isolated from the same tumor areas. In a few cases, this was not technically possible and cancer cells and stromal cells were then isolated from different tumor areas. For each sample, we estimated the number of cells isolated per lasershot (1–4 cells) and then calculated the number of lasershots required to isolate the desired number of cells. The same investigator performed all LCM dissections.

Extraction of RNA and construction of cDNA from LCM dissected samples

Lysis of LCM dissected cells and extraction of RNA was performed using the 'Absolutely RNA Nanoprep' kit with DNase digestion according to the manufacturer's instructions (catalog No. 400753, Stratagene, West Cedar Creek, TX). The RNA was eluted in 10 μl H_2O and ~7 μl of each RNA sample was used to produce cDNA for qPCR analyses. Specifically, 1 μl H_2O and 2 μg pd(N)6 random hexamers (Amersham Pharmacia, Buckinghamshire, UK) were added to the RNA and this mixture was incubated at 70°C for 10 min. Reverse transcription was performed with the Superscript II reverse transcriptase (Invitrogen, Taastrup, Denmark) according to the manufacturer's instructions using 10 mM of each dNTP (Roche) and adding 1 μl RNasin RNase inhibitor (40 U/μl, Promega, Southampton, UK).

qPCR analyses

qPCR analyses were performed essentially as described previously (25) with the following modification. Since we did not know the concentration of cDNA in each individual sample, we used a fixed volume of cDNA: for 18S qPCR, we used 1/55 of each cDNA sample, whereas 1/11 was used for target gene qPCR. Specific primers and fluorogenic probes for all investigated MMP genes, except matriptase (MMP-7), and 18S rRNA are described in detail in ref. (25). For uPA, PAI-1 and matriptase, the following forward (F) primers, reverse (R) primers and probes (P) were used: uPA: F: GAA ACC CTA CAA TGC CCA CAG A; R: GAC AAA CTG CCT TAG GCC AAT C; P: CAC AAT TAC TGC AGG AAC CCT GAC AAC. PAI-1: F: CCG TCT CTG TGC CCA TGA T; R: GGC AGT TCC ACG ACG TCA TA; P: CTC AGA GCA ACA AGT TCA ACT ACA CTG AGT TCA CC. Matriptase: F: AGA TCT TTC TGG ATG CGT ATG AGA; R: GGA CTT CAT TGT ACA GCA GCT TCA; P: TTT ATC AGC CTG GCC AGC CAG GTG. Primers and probes were designed using Primer Express 1.0 Software (PE Applied Biosystems, Warrington, UK) and synthesized by PE Applied Biosystems. The cycle threshold (C_T) value was used as an indicator of the amount of target RNA in each sample as described (25). For all target genes, a C_T value >35 was considered to indicate zero expression of the given gene in the given sample. Standard curves for C_T value versus input RNA were prepared and used to determine the levels of starting RNA in individual samples. Normalized expression levels were then calculated using the starting levels of 18S rRNA in each sample to normalize for differences in total RNA content in the individual samples using the methodology described, in great detail, in ref. (25).

In situ hybridization

ISHs for MMP-2, -3 and -13 were performed essentially as described (24). Plasmids used for transcription of radiolabeled probes were for MMP-2, pm72kD containing the HaeIII (2051–2386) fragment (GenBank M84324) in pGEM3Z (26) and pKaA205 containing the 35–691 fragment in pBluescript KS+; for MMP-3, pKaA219 containing the 584–1183 fragment (GenBank X63162) in pCRII (Invitrogen) and pKaA223 containing the 1186–1817 fragment in pBluescript II KS−; and for MMP-13, pCLM11-485 and pCLM11-810 that contain the 485 and 810 bp EcoRI fragments of MMP-13 (27).

Statistical analyses

Mean relative cancer cell or stromal cell expression levels for each gene in each tumor were calculated. To compare cancer cell (C) and stromal cell (S) samples, we calculated the ratio of expression in C versus S (normalized expression in C/normalized expression in S). The distribution of these ratios was skewed, whereas we observed an approximately normal distribution of the ratios after square root transformation. The square root transformed ratios for the mean normalized expression levels were compared using the paired *t*-test. This test was not performed for MMP-13. *P*-values were adjusted for multiple testing using a Bonferroni correction. The intra- and inter-tumor variation of the square root transformed normalized expression levels was calculated for uPA, PAI-1, MMP-2, -11 and -14 and compared by the analysis of variance (ANOVA) method. This analysis was not performed for MMP-3 and -13. The SAS® software package (version 8.2; SAS Institute, Cary, NC) was used to manage the data and to perform all statistical analyses. The significance level was set to 5%.

Results

The combination of LCM and qPCR gives highly reproducible results

As we wanted quantitative measurements of protease expression in our samples, we established the precision of our methods initially. In individual samples, the mRNA levels were normalized in relation to the amounts of 18S rRNA. Therefore, we first examined the reproducibility of our 18S rRNA determinations. We chose to do this on liver tissue, from which highly homogeneous cell populations can be readily isolated. Minimal variation between the individual LCM-isolated cell samples would therefore be expected. We isolated four samples of ~2000 cells from each of two mouse livers. RNA was extracted, cDNA prepared, and qPCR analyses were performed to establish the amount of 18S rRNA in each of the samples. As shown in Figure 1A, we observed only a ~5% variation in 18S rRNA C_T values between these eight individual determinations. As 18S rRNA in all cells is present in vast excess over any mRNA species, and therefore should be much easier to measure reproducibly, we also found it important to determine the variability of mRNA measurements in LCM dissected samples. We therefore measured the levels of both 18S rRNA and MMP-3 mRNA, using for each of the RNAs, five parallel qPCR reactions on aliquots of a single cDNA preparation constructed from stromal cells that were microdissected from a primary PyMT-induced mouse tumor. MMP-3 mRNA was detected in much lower amounts than 18S

rRNA, but still with very little variation between the C_T values as shown in Figure 1B.

Tumor analyses: uPA, PAI-1, MMP-2, -3, -11, -13 and -14 are predominantly expressed by stromal cells

We isolated two primary tumors from each of the five 13–15-week-old female FVB-PyMT mice, all of which had visible metastases on the lung surface at the time of killing. From each of the 10 tumors, we isolated three cancer cell samples and three stromal cell samples, each containing ~3000 cells (see Figure 2 for a representative example). RNA was extracted from the 60 samples, cDNA was made and each sample was analyzed for 18S rRNA content by qPCR. Generally, the 18S C_T values for stromal cell samples were higher than those for the cancer cell samples, indicating that the cancer cell samples contained more RNA than the stromal cell samples. All 18S C_T values remained well within the linear range of the qPCR reactions. In a pilot experiment, we had determined that a minimal amount of 18S cDNA was required to enable a reproducible qPCR analyses of protease mRNA expression. Three stromal cell samples had less than this minimal amount of 18S cDNA and were therefore discarded. cDNA from the remaining 57 samples were used in qPCR analyses for uPA, PAI-1, MMP-2, -3, -11, -13 and -14. Results for individual mRNAs in a given sample were normalized to the 18S level in the same sample as detailed in the Materials and methods section. Based on the three cancer and three stromal samples we had isolated from each tumor, we calculated the mean mRNA level for each gene in the cancer and stromal compartments of each of the 10 tumors (Figure 3). As MMP-13 mRNA was only detected in few of the samples (7 out of 27 stromal cell samples and 2 out of 30 cancer cell samples), we did not include the MMP-13 results in our statistical analysis of expression levels. Nevertheless, a clear stromal expression pattern was observed in the few tumor samples expressing MMP-13 (Figure 3). For all other genes, we used paired *t*-tests to compare the normalized mRNA levels that we observed in cancer cell versus stromal cell samples. Significantly higher mRNA levels were found in stromal cells than in cancer cell samples for all tested genes, i.e. uPA, PAI-1, MMP-2, -3, -11 and -14 (Figure 3). In one tumor (d1), uPA and MMP-14 mRNA levels were highest in the cancer cell samples (see Figure 3). This pattern was seen in all the three individual sets of cancer and stromal cell samples that were isolated from this tumor (data not shown). As we had isolated samples from 10 different tumors and these tumors came from five individual, although genetically identical mice, we compared the intra- and inter-tumor variation in the mRNA levels of individual genes using the ANOVA method. Again, we did not include MMP-13 data (see above) and we excluded MMP-3 as well, because many of the cancer cell samples did not contain detectable

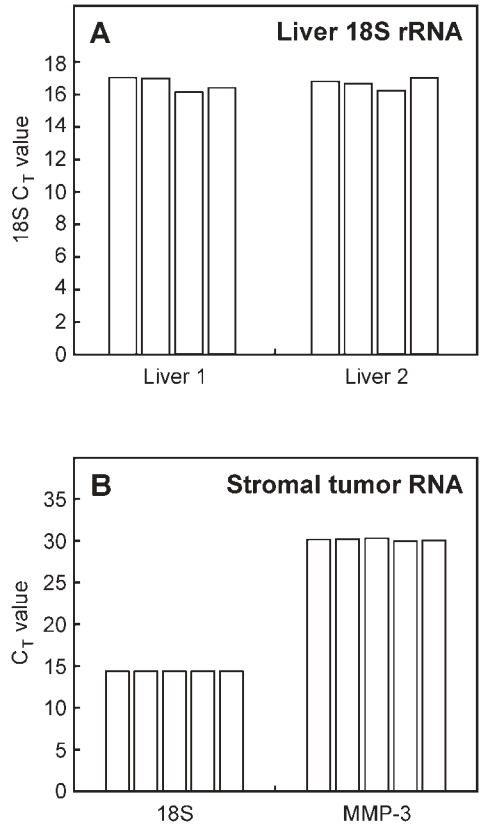

Fig. 1. Reproducibility of qPCR analyses. Cell populations were isolated from histological sections by LCM, total RNA extracted, cDNA prepared and qPCR performed. From each of the two wild-type mouse livers, four samples were analyzed for 18S rRNA (**A**). From a sample of stromal cells from a primary PyMT induced mammary tumor, 18S rRNA and MMP-3 mRNA levels were analyzed, using each cDNA template five times (**B**). Results are shown as C_T values. It should be noted that a higher C_T value indicates a lower starting level of RNA. An increase of 1 in the C_T value reflects a 2-fold decrease in the starting level of RNA.

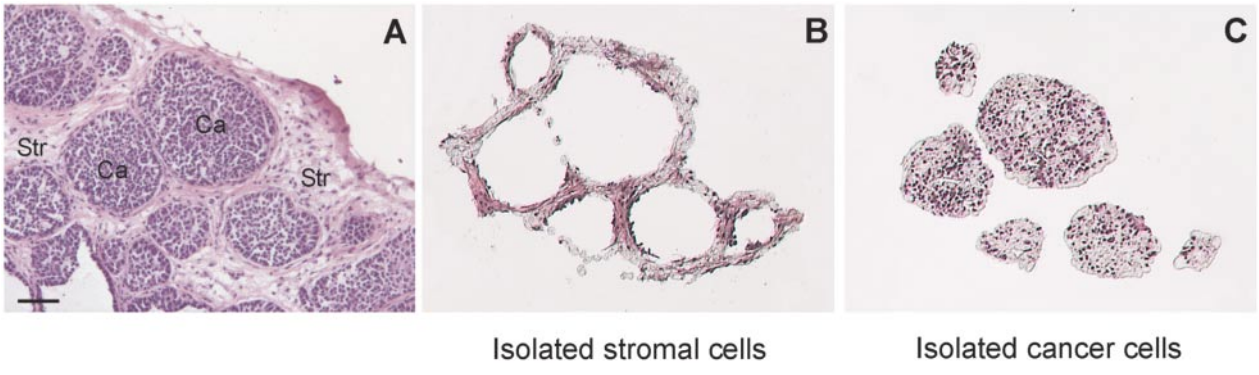

Fig. 2. LCM of cancer cells and stromal cells from a primary PyMT tumor. The cells were microdissected from a H/E stained section of a PyMT tumor (**A**) revealing well defined lobules of cancer cells (Ca) surrounded by stromal cells (Str). Images of the cells that are transferred to the polymer film by the laser beam show the isolated stromal cells (**B**) and cancer cells (**C**). Scale bar in (A): 100 μm.

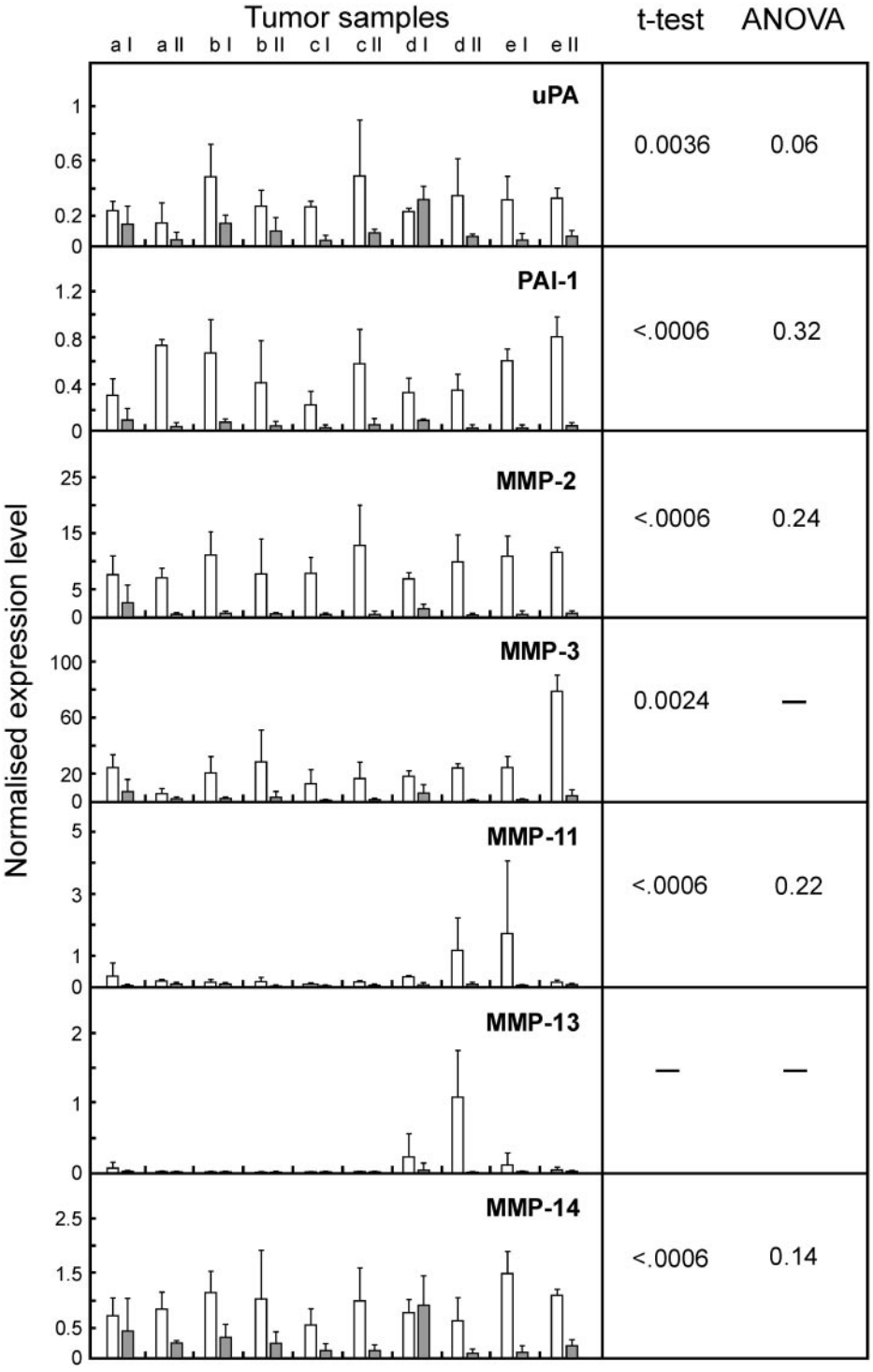

Fig. 3. qPCR analyses for protease expression in LCM dissected samples of cancer and stromal cells from primary PyMT tumors. Samples from two primary tumors (I and II) from each of the five different mice (a, b, c, d and e) were analyzed. The expression levels normalized with respect to the 18S rRNA levels were calculated for each of the indicated genes. The means of the levels in three samples of stromal cells (open bars) and cancer cells (shaded bars) are indicated. Error bars indicate SD. It should be noted that the absolute values of these expression levels are not directly comparable for different genes. For each gene is also indicated the statistical significance of the difference between the gene's normalized expression in stromal and cancer cells, as determined by paired *t*-tests (stromal expression was the highest for all the genes), and the significance levels for ANOVA tests of intra- versus inter-tumor variation in the expression levels.

MMP-3 mRNA. Therefore, the MMP-3 mRNA levels were not normally distributed and the ANOVA test was not applicable. For uPA, PAI-1, MMP-2, -11 and -14 mRNA levels, the variation in cancer/stroma mRNA ratios in samples isolated from a given tumor was not different from the variation observed in samples isolated from different tumors (Figure 3).

As we found a predominant stromal expression of all the seven investigated protease genes, we wanted to demonstrate that this was not caused by a hypothetical qPCR-inhibitory

activity present in cancer cell samples only. We therefore mixed a cancer cell cDNA sample with a stromal cell cDNA sample and performed MMP-3 qPCR analysis on the cancer sample, the stromal cell sample and the mixed cancer/stromal sample. As seen in Figure 4A, we detected a high MMP-3 mRNA level in the stromal sample, a low level in the cancer sample and an intermediate level in the mixed sample and therefore concluded that the cancer cell sample did not contain specific inhibitory activities. Finally, to test whether our method was also applicable to protease gene products that are primarily expressed in cancer cells, we analyzed the mRNA levels for the serine protease matriptase, which has been shown to be expressed by cancer cells in human breast cancer (28). For this analysis, we pooled aliquots of three cancer and three stromal cell RNA samples from three of the tumors to synthesize new sets of cancer and stromal cDNAs. These sets were used for matriptase and MMP-2 qPCR analyses. We found matriptase mRNA mainly in cancer cells, while MMP-2 mRNA was mainly found in stromal cells as previously seen (Figure 4B).

In situ hybridization

ISH analyses are not quantitative, but they show the exact cellular localization of specific mRNAs. We therefore did ISH analyses for the MMP-2, -3 and -13 mRNAs in order to compare the direct localization with our qPCR results. A clear stromal expression pattern of all three MMP genes was seen in the ISH analyses, while we did not detect any positive signals within cancer cell islands (Figure 5). MMP-2 mRNA was detected in 8/8 investigated PyMT tumors, uniformly distributed throughout the stroma in both the interior and the periphery of the tumors (Figure 5A–C). We found MMP-3 mRNA to be focally expressed in specific areas of the tumor stroma in 3/3 investigated PyMT tumors (Figure 5D–F). MMP-13 mRNA was detected in stromal cells in 9/9 tested PyMT tumors (Figure 5G–I), but with substantial differences among different tumors. In 4/9 tumors, only a sporadic expression was seen, whereas a high expression was detected focally in larger areas in the remaining five tumors. Thus, for all the three analyzed MMPs, the qualitative results of the ISH analyses are in good agreement with the quantitative results obtained using LCM combined with qPCR analyses (cf. Figures 5 and 3). In particular, stromal expression of MMP-2, -3 and -13 is seen with both methodologies.

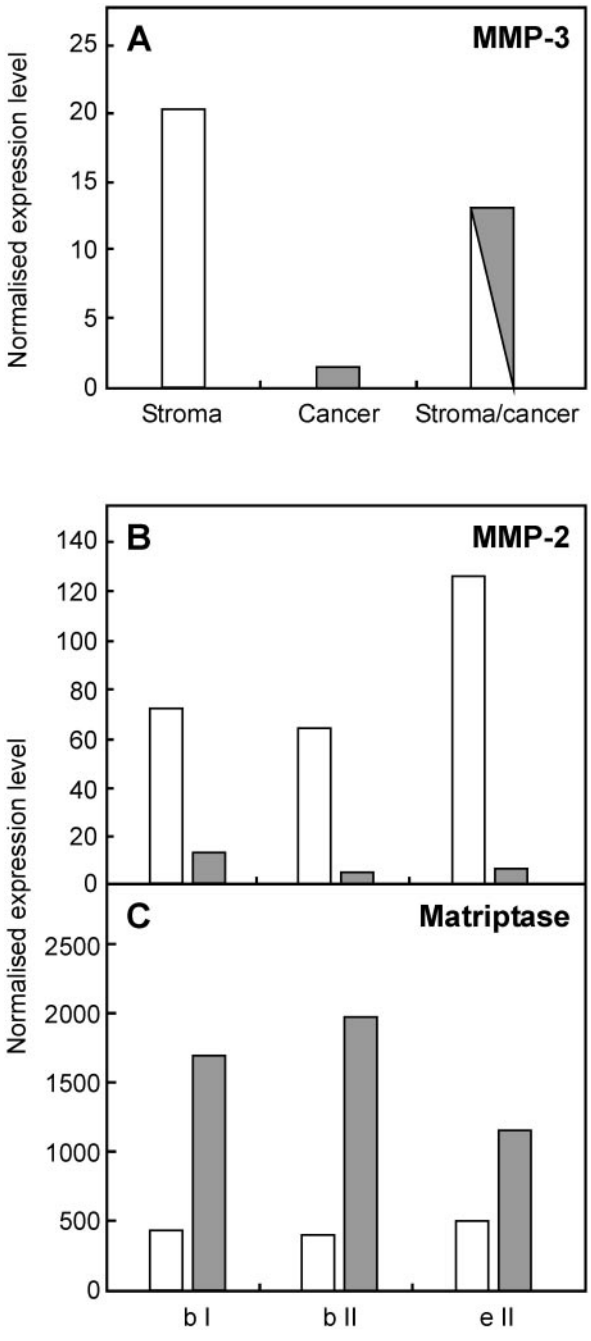

Fig. 4. qPCR analyses of a mixed cancer/stromal sample and for an epithelially expressed gene. Expression of MMP-3 was analyzed in a stromal cell sample and a cancer cell sample from a primary PyMT tumor and in a 1:1 mixture of the two samples (**A**). Expression of MMP-2 and matriptase was analyzed in pooled stromal cell (open bars) and cancer cell (shaded bars) samples (**B** and **C**) isolated from three primary tumors bI, bII and eII described in Figure 3. Bars indicate the gene expression levels normalized with respect to 18S rRNA levels. It should be noted that the absolute values of these expression levels are not directly comparable for different genes.

Discussion

We have shown that it is possible to combine LCM with qPCR analyses to obtain reproducible, quantitative measurements of gene expression levels in homogeneous cell populations isolated from heterogeneous tissue sections both for RNA present at high (18S rRNA) and low (e.g. MMP-3 mRNA) levels. We determined the mRNA levels for seven proteolytic components in 10 individual primary mammary tumors isolated from five different FVB-PyMT mice. From these tumors, we microdissected a total of 30 cancer cell samples and 30 stromal cell samples. For six of the mRNAs analyzed, those encoding uPA, PAI-1, MMP-2, -3, -11 and -14, qPCR analyses showed significantly higher levels in stromal cells than in cancer cells. The fact that this observation was not owing to some systematic bias for obtaining a stromal expression pattern by our procedure was shown by an analysis of matriptase mRNA, which was mainly found in cancer cell populations. Thus, we were able to identify genes predominantly expressed in stromal cells as well as a gene predominantly expressed in cancer cells. Cancer cell samples were nearly devoid of mRNA for PAI-1, MMP-2, -3 and -11, whereas we found uPA and MMP-14 mRNA in some cancer cell samples, although the amounts of these mRNAs in all but one tumor (tumor d1) were higher in stromal cells. Our qPCR results for MMP-2 and -3 were further supported by

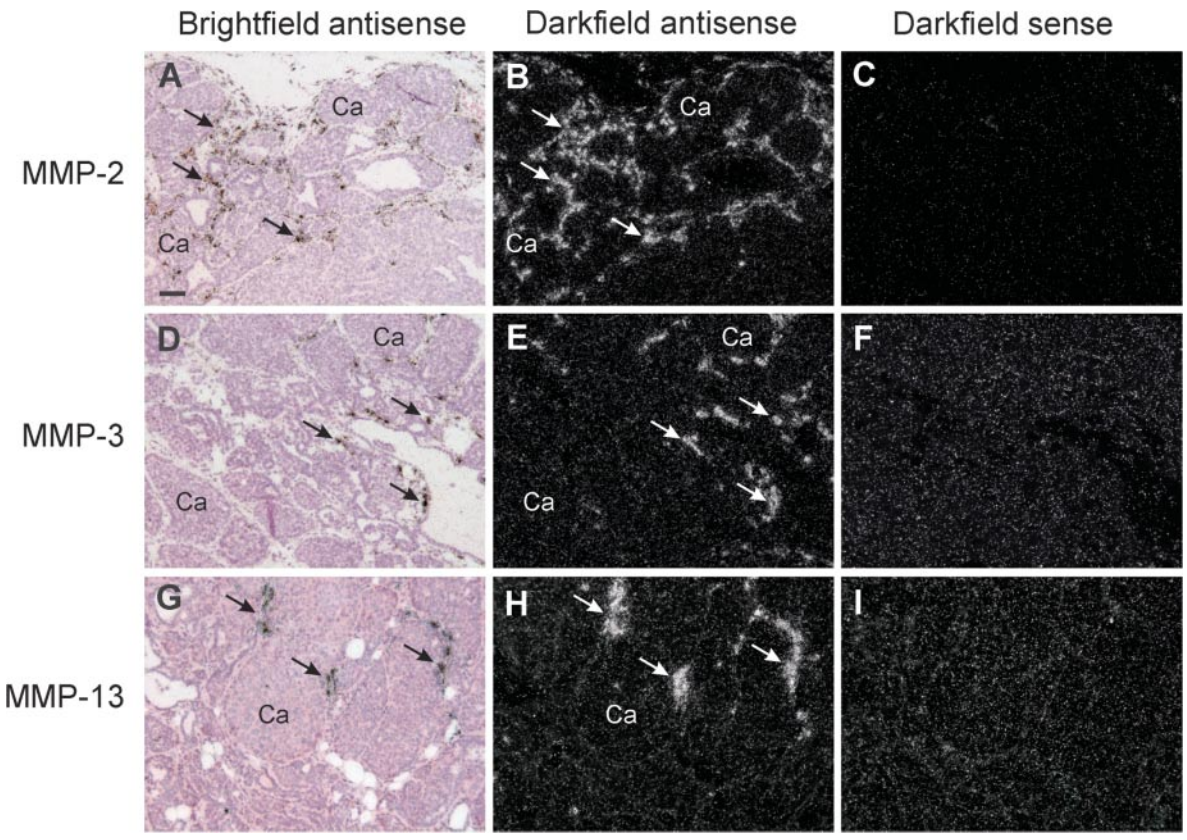

Fig. 5. ISH analysis of PyMT tumors. ISH with ^{35}S-labeled RNA probes was performed on primary PyMT tumors with antisense probes (**A**, **B**, **D**, **E**, **G**, **H**) or sense probes (**C**, **F**, **I**) complementary to MMP-2 (A–C), MMP-3 (D–F) or MMP-13 (G–I) mRNA. Representative H/E stained bright field (A, D and G) and dark field (B, C, E, F, H and I) sections are shown. 'Ca' indicates well-defined cancer cell islands surrounded by streaks of stromal cells. Arrows indicate positive signals for either MMP-2, -3 or -13 [seen as silver grains in bright field images (A, D and G) and white reflections in dark field images (B, E, and H)]. Scale bar in A: 50 μm.

ISH analyses, which showed a clear stromal expression pattern of both genes.

Previous ISH analyses of the expression patterns for uPA and PAI-1 in PyMT tumors also correlate well with the qPCR results in the present study. By ISH analyses, PAI-1 mRNA was found only in stromal cells in primary PyMT tumors (23), whereas uPA mRNA was detected both in stromal cells outside cancer cell islands and in a small population of unidentified single cells located within cancer cell islands (19). Since MMP-13 mRNA was undetectable in the microdissected cells obtained from the majority of the tumors in this study, we were unable to do a meaningful statistical analysis on the qPCR data. In those tumors that did express MMP-13, the mRNA was found mostly in stromal cell populations. This is in accordance with our ISH findings. Furthermore, the rather large variation in the normalized mRNA levels of both MMP-3 and -13 observed in the qPCR analyses between samples from individual tumors is in accordance with the pronounced focalized expression of these two MMPs observed by ISH.

Since our cancer cell preparations were practically without PAI-1, MMP-2, -3, -11 and -13 mRNA, we believe that we did not have a general problem with any stromal cell contamination of our cancer cell preparations. Immunohistochemical analyses have indicated that cancer cell islands in PyMT tumors are not infiltrated by α-smooth muscle actin-positive myofibroblasts or vimentin-positive mesenchymal cells (unpublished results), whereas F4/80-positive macrophages are invariably found both outside of and within cancer cell islands in PyMT tumors [(29) and unpublished results]. Thus, we expect that the isolated cancer cell populations contained some macrophages, but only insignificant numbers of vimentin-positive mesenchymal cells and myofibroblasts. The uPA and MMP-14 mRNAs detected in the cancer islands may therefore, at least partly, stem from tumor-infiltrating macrophages. The finding of higher mRNA levels of uPA and MMP-14 in the cancer cell population in one tumor (d1) compared with the stromal cell population may reflect an increased infiltration with macrophages in this particular tumor. We expect that the isolated stromal cell samples contained very few cancer cells, since the cancer cells are easy to recognize visually and are rarely seen in the areas where we microdissected stromal cells. Finally, the unambigous results we obtained in the statistical analyses of the qPCR data indicate that we do not have major contamination-derived problems in establishing cancer versus stromal cell expression patterns.

Analyses of the intra- versus inter-tumor variation in cancer/stromal expression levels indicated a high degree of similarity between the expression of uPA, PAI-1, MMP-2, -11 and -14 among samples isolated from 10 different tumors. The 10 tumors were isolated from five different, but genetically identical mice, and we did not observe any expression patterns that were specific for individual mice. The relatively small tumor–tumor and mouse–mouse variation suggests that the expression levels of the investigated genes in this transgenic

mouse model are reproducible. In accordance with these findings, a microarray study has shown that the global gene expression patterns observed in tumors isolated from different PyMT mice are very similar (30). The consistency in expression patterns is an important quality of the PyMT model, which makes analyses of the model more robust.

Generally, the expression results we have obtained using the transgenic PyMT model are in good agreement with previous results observed in human ductal mammary carcinomas for uPA, PAI-1, MMP-2, -3, -11, -13, -14 and matriptase. Apart from matriptase, mRNA for all these components have been detected by ISH in stromal cells, most notably fibroblast-like cells, in human breast cancer (10–13,15–17,31). In contrast, matriptase mRNA has been detected by ISH both in cancer cells and surrounding normal breast epithelial cells, but not in stromal cells, in primary human breast cancer (28).

The fact that tumor-associated stromal cells express components of the extracellular proteolytic machinery suggests that the cancer cells recruit stromal cells to produce these components (1,2). A number of different signaling molecules that might participate in such recruitment of stromal cells have been proposed (reviewed in ref. 3). For example, transforming growth factor-β (TGF-β) is expressed by cancer cells in breast cancer (32) and TGT-β can induce the differentiation of normal mammary fibroblasts to myofibroblasts, a characteristic stromal cell type in many carcinomas (33). In addition, blockade of TGF-β signaling in FVB-PyMT mice has been shown to reduce MMP-2 and -9 activity in extracts of primary tumors and to decrease lung metastasis (34). Another example is the glycoprotein emmprin (extracellular matrix metalloproteinase inducer), which is expressed by some types of cancer cells and can induce fibroblasts and endothelial cells to produce several MMPs (35).

However, much work remains to be done to obtain an insight into the mechanisms by which cancer cells recruit and direct stromal cells. With respect to proteolysis and stromal cell involvement, we have previously proposed that cancer invasion mimics specific remodeling processes in the tissues from which the cancers originated. Thus, ductal mammary carcinoma appears to mimic postlactational mammary gland involution and squamous cell skin cancer to mimic skin wound healing. It may therefore be fruitful to view cancer invasion as tissue remodeling processes gone out of control (1,2,24). The methodology described here allowing a simultaneous quantitative determination of mRNA levels for both proteolytic components and candidate signaling molecules, as well as their receptors in specific populations of cancer and stromal cells, may prove to be a useful tool in further exploring similarities and differences between cancer invasion and non-neoplastic tissue remodeling processes.

The involvement of stromal cells in extracellular proteolysis and thereby in tumor progression has important implications for the biology, as well as the prevention and therapy, of cancer. Recruitment of stromal cells is likely to be crucial both for carcinogenesis and for establishment of metastases, and stromal cells are thus attractive targets for new therapeutic approaches (1,2,4,36). Transplanted tumors do not usually mimic human cancers in this respect and are therefore poor models for experimental studies of the role of stromal cells (37). In contrast, the predominant stromal cell expression of protease components found in the present study indicates that the PyMT-induced mammary cancer is an attractive model to be used for such studies.

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Conflict of Interest Statement: None declared.

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