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Selective expression of a splice variant of decay-accelerating factor in c-erbB-2-positive mammary carcinoma cells showing increased transendothelial invasiveness

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Abstract

By differential-display-PCR a subclone of the SK-BR-3 cell line with high in vitro transendothelial invasiveness was identified to express increased levels of a new alternative splice variant of decay-accelerating factor (DAF). DAF seems to play an important role in some malignant tumours since on the one hand the expression of complement inhibitors on the surface of tumour cells prevents the accumulation of complement factors and in consequence cell lysis. On the other hand, DAF has been identified as a ligand for the CD97 surface receptor which induces cell migration. Immunofluorescence procedures, Western blot analyses, and cDNA clone sequencing were employed to confirm the expression of DAF restricted to invasive tumour cells. Using a radioactive RNA-in situ hybridisation on freshly frozen tissue microarrays and RT-PCR on native tumour tissue, the expression of alternative spliced DAF mRNA was demonstrated in invasive breast cancer. Due to the fact that it could thereby not be detected in normal mammary tissues, it has to be confirmed in larger studies that the DAF splice variant might be a specific tumour marker for invasive breast cancer.

Keywords: Decay-accelerating factor alternative splice variant; c-erbB-2; Breast cancer; Differential expression; Invasion

Using an in vitro invasion model based on the migration of tumour cells through an endothelial layer, cells with increased transendothelial invasiveness could be selected and cultivated from an unselected culture of the c-erbB-2-positive mammary carcinoma cell line SK-BR-3 [1]. Here, this subclone of cells was studied in more detail for its properties relating to c-erbB-2 overexpression that conveys a higher invasive potential.

Our starting point was a comparison of the mRNA expression profile of the invasive subclone with that of the entire cell population of the cell line SK-BR-3 using *differential-display*-PCR. Using this procedure it could be shown that an alternative splice variant of the decay-accelerating factor (DAF), also known as CD55, is overexpressed in SK-BR-3 cells with increased transendothelial invasiveness. Immunofluorescence procedures, Western blot analyses, and cDNA clone sequencing were employed to confirm this differential expression in more detail. Finally, using a radioactive RNA-in situ hybridisation (RISH) on freshly frozen "tissue-microarrays"

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(TMA) and RT-PCR on native tumour tissue, the expression of alternative spliced DAF mRNA was also demonstrated. Thereby, we were able to show that different splice variants of DAF are expressed also in mammary carcinomas in vivo. The alternative splice variant of this protein, due to the fact that it could not be demonstrated in normal mammary tissue, ought to be investigated in further studies.

Materials and methods

Breast cancer cell line SK-BR-3 was obtained from the American Type Culture Collection (ATCC, Rockville, MA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (ICN, Eschwege, Germany) supplemented with 2 mM L-glutamine, antibiotic drugs as above, and 10% FCS. The invasive subclone was obtained from the SK-BR-3 cells which could penetrate the extravasation model. The model system contained human umbilical vein endothelial cells (HUVEC) which were grown on gelatin-coated flasks and passaged four to six times in a medium containing equal volumes of Iscove's modified Dulbecco's medium (IMDM) and Ham's F12 nutrient mixture (Life Technologies, Eggenstein, Germany) supplemented with 10 μg/ml sodium heparin (Boehringer Ingelheim, Heidelberg, Germany), 5 μg/ml transferrin, 2.5 ng/ml basic fibroblast growth factor (bFGF) (Sigma, Deisenhofen, Germany), 5 μM β-mercaptoethanol, 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B (Sigma), and 15% fetal calf serum (FCS) (PAA Laboratories, Linz, Austria).

The invasion assays were performed as described by Roetger et al. [1]. In brief, cell culture inserts with 8 μm porous polyethylene terephthalate (PET) membranes were coated with basement membrane extracellular matrix (ECM) (Harbor Bio-Products, Norwood, MA, USA) at a concentration of 125 $\mu g/cm^2$ by drying an appropriate ECM dilution overnight under a laminar flow hood. HUVEC were seeded onto the rehydrated coated membranes in a concentration of 2×10^5 cells/well. After confluent monolayer formation, 2×10^5 SK-BR-3 cells were placed onto the HUVEC monolayer on the ECM-coated membrane. The invasion assays of primary breast cancer cells were performed applying approximately 10^6 disaggregated cells to the membrane. The invasion medium was placed into the wells under the bottom sides of the membranes as well. Invasion assays were incubated for 48 h, and thereafter, HUVEC monolayer and non-invading cells on the upper surface of the membrane were removed. Invading cells on the bottom side of the membrane were cultured as described above.

For differential-display-PCR total RNA from SK-BR-3 cell lines were isolated with RNAzol B reagent (Biotex Laboratories, Houston, TX). Two-base anchored oligodesoxythymidylate primers $HT_{11}G$, $HT_{11}A$, and $HT_{11}C$ were used to reverse transcribe RNA from SK-BR-3 cells into first-strand cDNAs, which were amplified subsequently by PCR using the arbitrary upstream primer HAP-10 5′-AAGCTTCCACGTA-3′ of the RNAimage Kit 1 und 2 (GenHunter, Nashville, TN). PCR conditions used were the same as described previously. PCR products were analysed on a 6% DNA sequencing gel using 0.5 mM α-^{35}S-labelled dATP (1200 Ci/mmol). The bands on the cDNA ladder that were unique to SK-BR-3 parental cell line or to the invasive subclone were cut of the gel, eluted, and reamplified by PCR.

For cloning and sequencing the reamplified cDNA bands were cloned into plasmid PCRII using TA cloning kit (Invitrogen, San Diego, CA). Individual clones were sequenced using an automated DNA sequencer (Applied Biosystems, Weiterstadt, Germany). The sequence was compared to that of NCBI *Homo sapiens* decay-accelerating factor for complement (CD55, Cromer blood group system) (DAF), mRNANM_000574 (2308 bp, 20-DEC-2004). This sequence was used furtheron for primer, probe design, and sequence comparison.

Differential expression of EPIL mRNA in SK-BR-3 parental cell line versus the invasive subclone was confirmed using PCRII plasmids carrying the specific sequence of EPIL as a probe in quantitative ribonuclease-protection assay (RPA). RPA was performed by non-radioactive HybSpeed RPA kit according to the manufacturer's instructions (Ambion, Austin, TX).

For immunofluorescence studies the cells were fixed and permeabilised, and blocked with 10% human AB-serum (AB-serum for serological reactions, Biotest, Dreieich, Germany) to inhibit non-specific staining. The cells were further incubated with the following antibodies: rabbit polyclonal anti-c-erbB-2 antibody (0485, Dako, Hamburg, Germany). The mouse antibody and the rabbit antibodies were visualised using polyclonal Alexa 594-conjugated sheep-anti-mouse IgG F(ab′) fragments and FITC-conjugated goat-anti-rabbit antiserum, respectively, which were applied for 30 min at room temperature. After rinsing the slides with PBS, staining was evaluated by a fluorescence microscope Laborlux S (Leica, Wetzlar, Germany).

For sequencing of DAF-cDNA from SK-BR-3 cells with raised transendothelial invasiveness, mRNA was isolated using the mRNA direct kit (DYNAL, Oslo/NOR). *Taqman reverse transcription reagents* (Applied Biosystems, Weiterstadt, Germany) were used for reverse transcription. The subsequent amplification of the cDNA was carried out using Ampli*Taq* Gold DNA-polymerase (Applied Biosystems, Weiterstadt, Germany); primer sequences: forward, CACCAC CTGAATGCAGAGGAA and reverse, CCTCCCTTATCACCATC AACACC. This primer and the PCR protocol were also used for the detection of the alternative splice variant in native tumour tissue. For cloning the PCR products the Original TA Cloning Kit (Invitrogen, San Diego, USA) was applied. The plasmid mini preparation was carried out using QIAprep Miniprep Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. The cloned fragments were sequenced using the reagents of the "ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits" (ABI) (Applied Biosystems, Weiterstadt, Germany) and fractionated by capillary electrophoresis using the ABI Prism 3700 capillary sequencer (Applied Biosystems, Weiterstadt).

For positive controls a portion of the DAF-cDNA was applied to an agarose gel for gel electrophoresis. After separation, the marked DNA probes that were generated were transferred at 68 °C to a nylon membrane upon which they were detected using DIG RNA labelling kits (Roche, Basel/SWI).

For the detection of DAF mRNA the radioactive RNA-in situ hybridisation on "fresh-frozen" tissue microarrays (RISH) was applied. The sequences of the oligonucleotide probes ($\geqslant$40mer) for the DAF-mRNA were identified using the Vector NTI program (Infor Max, Invitrogen life science software, Oxford, UK) on the cDNA sequence NM_000574 (2308 bp, mRNA, linear, PRI 20-DEC-2004) before they were commercially synthesised. Two oligonucleotide probes were employed for this purpose to sequences in 5′–3′ direction: 5′ probe, CTTGTTGGCACCTCGCAGCTACGATTGCAGAACTCT TCA; 3′ probe, CCTCCCTTATCACCATCAACACCCCTGGTTC ACCAGCATGTT. All of the complementary oligonucleotides against their respective DAF-mRNAs were first marked separately using a radioligand "3-tailing"-reaction. Then, the matching probes were pooled and purified (Qiagen nucleotide removal kits; Qiagen, Basel, Switzerland). The marking efficiency was checked in a Packard Tri-Carb liquid scintillation counter (Perkin-Elmer, Boston, MA, USA). In order to guarantee a satisfactory signal intensity, only radioactive markings showing an activity of at least 100,000 cpm/μl probe were used for hybridisation. In addition, frozen TMAs were employed for the RISH. Each TMA-array contained tissue from 230 invasive mammary carcinomas (193 invasive-ductal, 29 invasive-lobular, 7 medullary, 1 mucinous, and 1 tubular carcinoma with known follow-up data in each case) and 10 normal mammary tissue samples as

controls. For analysing a TMA-array, 120 μl of hybridisation solution was added together with enough radioactively marked probes to achieve an activity of 5,000,000 cpm/ml hybridisation solution (1 h at 42 °C). After that the solutions were applied to the TMA-array and hybridised in a humid chamber (overnight at 42 °C). After the hybridisation process, the samples were washed, dehydrated, and air-dried. The radioactive signals of the hybridised RNA were visualised after a 48 h exposure to a high-resolution "phosphorimaging screen" (Packard Canberra, Zurich, Switzerland) using the "Cyclone Phosphorimager" (Packard, Tokyo/Japan). The "ArrayVision software package" (Imaging Research, Ont., Canada) was used for image analysis. An activity of $\geqslant$180,000 cpm was defined as a strong expression.

For the detection of the splice variant in vivo mRNA isolated from 23 DAF-pos. tumours was applied to the PCR protocol as mentioned for sequencing.

Results and discussion

Identification of an alternative splice variant of decay-accelerating factor in SK-BR-3 cells of different transendothelial invasiveness and breast cancer tissue

Using the above-described in vitro invasion model [1] a subclone could be selected and cultivated from the total cell population of the cell line SK-BR-3 which showed an increased transendothelial invasiveness. From both populations the mRNA was then isolated and differences in the expression of individual genes were detected using *differential-display*-PCR. DNA strands that allowed a differential mRNA expression in the two cell populations to be concluded were detected directly by sequencing. Using the random primers $HT_{11}C$ (oligo(dT)-"primer") and HAP-10 (random "primer") with the human gene for the, a gene was identified, in the form of the "decay-accelerating factor" (DAF), that was differentially expressed in the two cell populations.

By using a semi-quantitative " ribonuclease-protection assay " (RPA) it could be shown that the DAF-mRNA is overexpressed in the cells of the population with increased transendothelial invasiveness. However, the DAF-mRNA-fragment verified in the RPA contained only about 200 bases; after cloning and sequencing of the amplified cDNA, 308 bases had in fact been expected (Fig. 1).

Immunocytochemistry using anti-CD55 monoclonal antibody revealed only minor differences in DAF expression between the cells with increased transendothelial invasiveness and the total SK-BR-3 cell population. A striking feature of the invasive cell clone, however, was that a large proportion of the protein was localised in the cytoplasm (Fig. 2). In healthy cells, such as those involved in hematopoiesis, DAF is usually anchored to the cell membrane via a GPI anchor. An arrangement of the protein along the cell membrane, such as that seen for c-erbB-2 receptors in the control stains, was not found in the tumour cells for the most part.

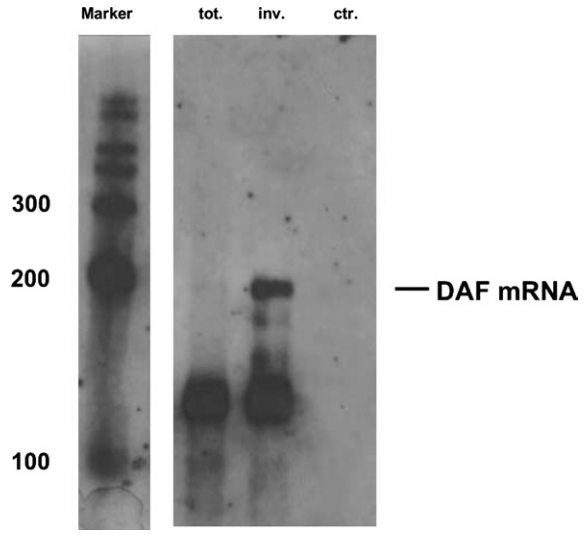

Fig. 1. Identification by DD of altered gene expression in parental SK-BR-3 cells and invasive subclone. Confirmation of the results by ribonuclease-protection assay (RPA). Assignment of the lanes: marker, RNA size marker; tot., DAF-mRNA from the cells of the total cell population of the cell line SK-BR-3; inv., DAF-mRNA from the cells of the invasive subclone; ctr., control run with yeast RNA.

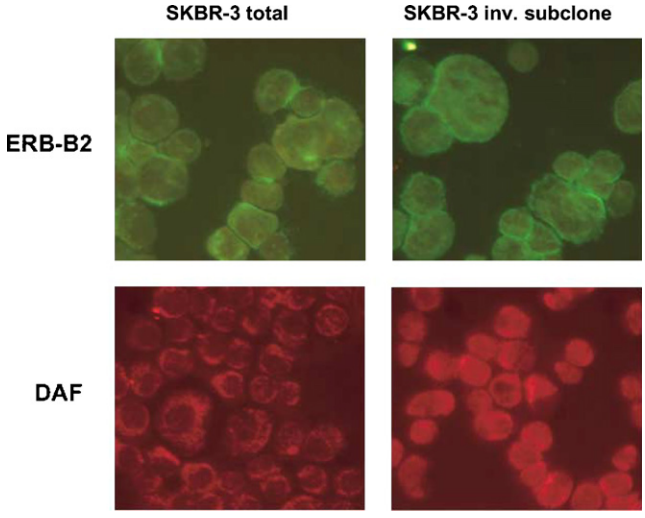

Fig. 2. Immunocytochemical demonstration of the decay-accelerating factor (red fluorescent staining below) as well as the c-erbB-2 receptor (green immunofluorescent staining, above) in cells of the SK-BR-3 total cell population (to the left) and the invasive subclone: It is striking that in the cells of the invasive subclone a large proportion of the DAF is localised in the cytoplasm, since DAF is usually anchored via a GPI anchor to the cell membrane. In the two upper pictures on the other hand the c-erbB-2 receptor can be visualised in the area of the cell membrane as expected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Western blot analyses revealed that the two examined cell populations of the cell line SK-BR-3 expressed the decay-accelerating factor (DAF) in different molecular weight forms. In the cells of the total cell population, a variant with a molecular weight of 70 kDa was predominantly expressed, but in the cells of the invasive clone a second DAF isoform with a molecular weight of approx. 45 kDa was also very strongly expressed (Fig. 3). This DAF isoform appeared also in the culture medium of the invasive subclone and did not appear to occur at all in the cells of the total cell population.

DAF can be demonstrated in the cells of many different tissues. This protein occurs in a number of isoforms of different sizes. This is due on the one hand to different post-translational modifications (e.g., glycosylation [2]). However, as has been shown already on many occasions, different DAF isoforms can also arise from alternative splicing in the cells of a number of tissues [3]. After we demonstrated this differential protein expression, we then intended to sequence the DAF-DNA from the SK-BR-3 cell population with increased transendothelial invasiveness in order to provide clues as to whether this process might actually be responsible for the synthesis of the differently sized DAF variants.

The sequencing of the DAF-cDNA showed that in SK-BR-3 cells of increased transendothelial invasiveness two different forms of DAF-mRNA are expressed. In the second variant, a part of the sequence comprising 320 bps was deleted (Fig. 4).

In order to determine that DAF plays a role not just in cells of the cell line SK-BR-3 but also in invasive mammary carcinomas the total mRNA expression was measured in breast cancer tissues. In all, 230 invasive mammary carcinomas were studied by radioactive RNA-in situ hybridisation (RISH) on freshly frozen "tissue microarrays" (TMA); the arrays were assessed

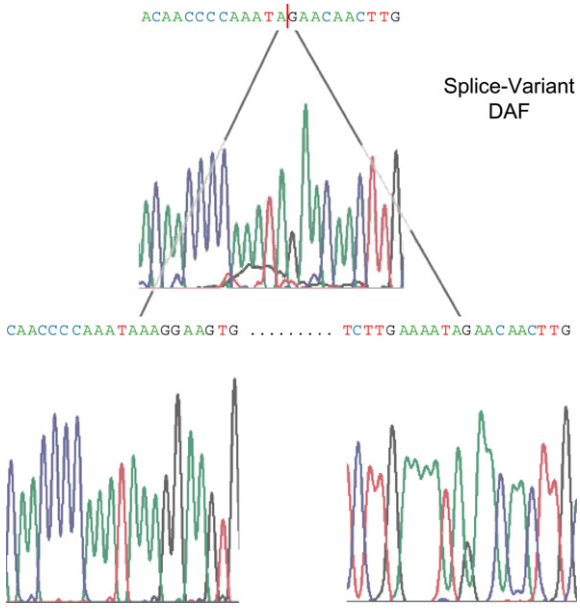

Fig. 4. Result of the sequencing of the DAF-cDNA from the invasive subclone of the SK-BR-3 cell line: (above) the portion of the abridged DAF-cDNA sequence in which the alternative splicing occurs as well as the 5′ (below to the left) and 3′ (below to the right) ends of the longer DAF-cDNA sequence are represented. The diagonal black lines in each case depict the positions of the alternative splicings. RNA was isolated as starting material from the SK-BR-3 cells of the selected invasive subclone.

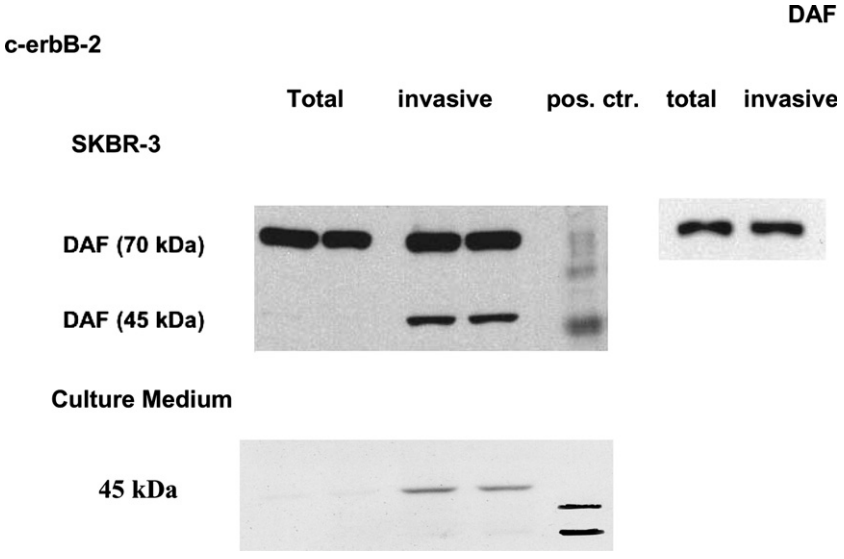

Fig. 3. Western blot analysis: illustration of the differential expression of decay-accelerating factor (DAF) in the cells of the total SK-BR-3 cell population (SK-BR-3 total) and the selected invasive subclone (SK-BR-3 invasive) using Western blot. As a positive control a cell lysate of erythrocytes was also analysed that also expresses DAF under physiological conditions (positive control [Erythr.]). In the right part of the picture, the cellular expression of *c-erb*B-2 by the cell line SK-BR-3 visualised by Western blotting is depicted. On the bottom differential expression of the DAF 45 kDa isoform was reflected by the detection of this protein in the culture medium of the invasive subclone.

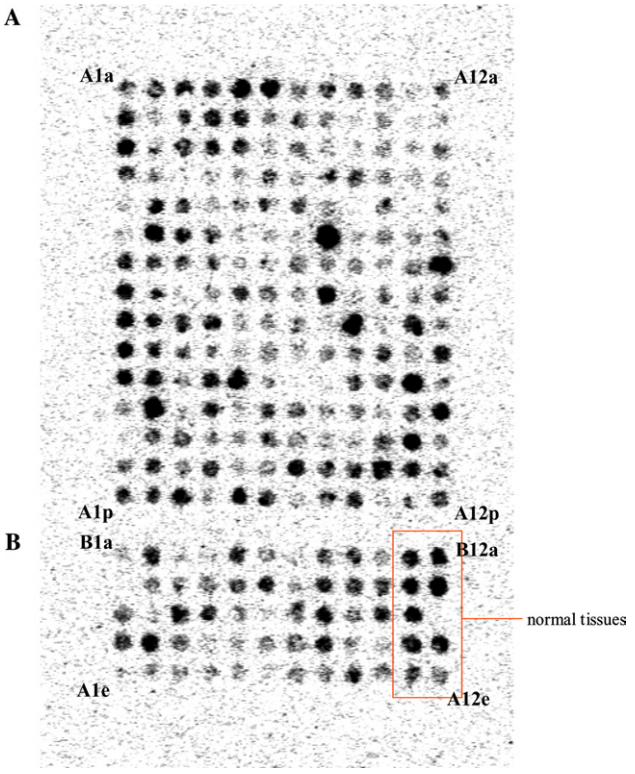

Fig. 5. Radioactive RNA in situ hybridisation (RISH) on freshly frozen tissue microarrays: tissue samples from 230 mammary carcinomas and 10 normal mammary tissues (outlined in black) were analysed for the expression of different DAF splice variants. Tissue samples with strong expression are depicted in black.

for the expression of the full-length mRNA using two probes located closer to the 5-prime and the 3-prime end of the cDNA. Expression of DAF mRNA was shown in 86 of the 230 examined carcinomas (Fig. 5). This was also found in 8 of the 10 examined tissue samples from normal mammary tissue. The alternative splice variant of DAF on the other hand could be verified only in 23 of 230 tumours by PCR. It was notable that tissue samples of healthy mammary tissue were all negative for this splice variant.

Discussion

Differential-display-PCR (*dd*-PCR) allowed the decay-accelerating factor (DAF) gene to be identified that appeared to be overexpressed in the cells of the invasive subclone of SK-BR-3. The results of the *dd*-PCR could be confirmed by ribonuclease-protection assays (RPA). However, it was interesting that the protected DAF-mRNA-fragment arising from the RPA contained only about 200 bases instead of the 308 that were expected.

Western blot analyses revealed that the cells of both cell populations contained about the same proportion of a 70 kDa isoform of the protein, while a 45 kDa variant was expressed almost exclusively in the cells of the invasive subclone and secreted to the culture medium. This might explain the fact that when applying immunofluorescent procedures, no large differences in DAF expression were seen between the two cell populations, and also the fact that the protected DAF-mRNA-fragment produced in the RPA contained only about 200 bases as opposed to the expected 308.

In order to clarify the mechanism responsible for bringing about the different isoforms in the two examined cell populations of the SK-BR-3 cell line, the DAF-cDNA from cells of the invasive subclone was sequenced. In this way, two different sequences could be identified. In the cells of the invasive SK-BR-3 subclone, an alternative splicing occurred which resulted in the production of a 45 kDa isoform of DAF.

With radioactive in situ hybridisation (RISH) on fresh-frozen tissue microarrays, oligonucleotide probes were employed for depicting transcripts of DAF total mRNA. It was found that DAF mRNA expression could be confirmed in a proportion of the tumours. It was notable that the larger splice variant was also found in 8 of the 10 healthy mammary tissue samples, while the smaller variant was only found by PCR in a few of the mammary carcinomas that were invasive. This might be a further sign that the expression of this splice variant of DAF might be associated with the progression of mammary carcinoma.

Expression of DAF has already been confirmed in tumour tissues and cell lines [2,4–8] as well as in metastases of malignant tumours [9]. In cells of colorectal carcinomas, various variants could be attributed to different glycosylation patterns [10], while in the present study alternative splicing was attributed as the cause for the origin of the 45 kDa isoform of DAF. The best known function of this protein is its effect as a complement inhibitor [11]. This function does indeed seem to play an important role in some malignant tumours since on the one hand the expression of complement inhibitors on the surface of tumour cells prevents the accumulation of complement factors, and on the other hand a blocking of these molecules with specific antibodies can render tumour cells more prone to complement-induced damage [6,12–15]. Also, DAF appears to exert an inhibitory effect on natural killer cells [16]. It is certainly conceivable that the expression of DAF and especially the 45 kDa splice variant of this protein verified in the tumour cells with increased invasiveness confers a kind of protection against attack by the human immune system.

The supposition that the expression of DAF in malignant tumours might be closely associated with an increased aggressiveness, invasiveness, and metastatic potential of the tumour cells can be presumed from the fact that DAF has been identified as a ligand for the CD97 surface receptor [17] that is presumed to play an essential role in the migration of neutrophilic granulocytes [18]. In thyroid gland and colorectal carcinomas

the expression of CD97 is correlated with an increasing dedifferentiation of the tumour cells, their potential for invasive growth and migration, and their ability to form lymph node metastases [18,19]. In colorectal carcinomas, CD97 is expressed intensively above all in the cells at the "invasive front" of the tumour [20]. The decay-accelerating factor (DAF) that is the ligand for CD97 can be synthesised by colorectal carcinomas and released into their surroundings [20]. Such tumour cells might also be capable of an autocrine stimulation of invasive growth, migration, and metastasisation. In other studies on malignant tumours, DAF was found to be present at higher levels in the tumour stromal [23]. Here, it was also presumed that the DAF originated from the tumour cells and that it was either cleaved from cell membranes or secreted in soluble form by the tumour cells [21,22]. A similar mechanism might also be conceived in the mammary carcinoma cells of the SK-BR-3 cell line. As such, the 45 kDa splice variant of DAF detected in the invasive cell population might represent an additionally synthesised and secreted isoform of this molecule and might in this way participate in the invasive growth of these cells as an autocrine stimulus.

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