PAM-1, a natural human IgM antibody as new tool for detection of breast and prostate precursors

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Abstract. Early detection and differential analysis of premalignant lesions are very important for both prognosis and therapy of cancer patients. A good source of diagnostic tools is the natural antibody pool of humans. Tumor-specific antibodies can be established by using hybridoma technology. The fully human germine-coded monoclonal IgM antibody PAM-1 was isolated from a patient with a stomach carcinoma. PAM-1 reacts with a post-transcriptionally modified isoform of membrane receptor CFR-1 which is overexpressed on almost all epithelial cancers of all types and origins.

The expression of CFR-1/PAM-1 on precancerous stages of breast and prostate cancer was analyzed by immunohistochemistry and compared with normal breast and prostate tissue as well as adenocarcinomas of both. In addition FACS analysis was performed to detect receptor expression on benign and malign prostate cells. 73 different tissue samples of prostate and breast precancerous stages and prostate and breast carcinomas were analysed for CFR-1/PAM-1 expression immunohistochemically. The CFR-1/PAM-1 receptor was expressed on nearly all precancerous stages and carcinomas while normal breast and prostate tissue showed negative results. These results were confirmed by FACS analysis showing a CFR-1/PAM-1 expression only on prostate carcinoma cells but not on benign prostate hyperplasia cells. The unique expression of this new CFR-1/PAM-1 receptor makes the PAM-1 antibody an ideal diagnostic and even therapeutic tool for precancerous and cancerous epithelial lesions of the breast and the prostate.

Keywords: Precancerous and cancerous lesions, isoform of CFR-1, human monoclonal antibody, diagnostic marker

Abbreviations: CFR-1 (cysteine-rich fibroblast growth factor receptor 1), DAF (decay-accelerating factor), D-CIS (ductal carcinoma in situ), L-CIS (lobular carcinoma in situ), PIN (prostate intraepithelial neoplasia), BPH (benign prostatic hyperplasia)

1. Introduction

Precancerous epithelial lesions are sites of uncontrolled cellular proliferation, generated by irreversible genetic changes. Not all these lesions progress to invasive cancer, some may even regress, but the early detection of abnormal cells can be crucial for patient survival. Most cancers and precursors are still diagnosed by pathologists based on subjective assessment of altered cell and tissue structure. Enlargement of the nucleolus is the only key diagnostic feature of high-grade prostatic intraepithelial neoplasia (PIN), an early stage that appears to be the precursor of the majority of invasive prostate cancers [1].

Diagnosis of the breast cancer precursor lesions D-CIS (ductal carcinoma in situ) and L-CIS (lobular carcinoma in situ) is mainly performed by determination of the nuclear grade of the lesion and the presence or absence of necrosis and cell polarization [2]. Al-
though pathological criteria have been established for the diagnosis of D-CIS distinguishing intraductal carcinoma from atypical ductal hyperplasia remains difficult. Nearly all established conventional immunohistochemical markers, like PSA, Ki-67, Her2/neu etc. are not tumor-specific or only expressed on a subset of PINs and D-/L-CIS and therefore not really predictive [3–5].

The fully human monoclonal IgM antibody PAM-1 was isolated from a patient with stomach carcinoma using the conventional human hybridoma technology by immortalizing lymphocytes from cancer patients. This technique offers the unique possibility to generate new fully human monoclonal antibodies for the diagnosis and therapy of diseases and, in addition, to characterize new tumor related membrane receptors with a single experimental approach [6–11].

PAM-1 binds to a new variant of the post-transcriptionally modified receptor CFR-1 and induces apoptosis [8,11,12]. The receptor is expressed on nearly all epithelial cancers of every type and origin, but not on healthy tissue. CFR-1 is also present on precursor lesions found in: Helicobacter pylori-induced gastritis, intestinal metaplasia and dysplasia of the stomach, ulcerative colitis-related dysplasia and adenomas of the colon, Barrett metaplasia and dysplasia of the esophagus, squamous cell metaplasia and dysplasia of the lung and cervical intraepithelial neoplasia [8,11]. In this study we present new data on the unique expression of this CFR-1/PAM-1 receptor on prostate precancerous lesions (PIN) as well as on breast cancer precursors (D-/L-CIS), showing that the PAM-1 antibody is an ideal reliable diagnostic (and therapeutic) tool for the detection of precancerous and cancerous lesions of prostate and breast.

2. Methods

2.1. Cell culture

The human hybridoma cell line 103/51 (PAM-1) was produced and grown as described previously [13].

2.2. Immunohistochemical staining of paraffin sections

Paraffin-embedded human tissue samples from 73 patients were sectioned (2 µm), deparaffinized and heated in citric acid (pH 5.5) in a pressure cooker for 5 min. Then the sections were blocked with bovine serum albumin (BSA, 5 mg/ml) diluted in phosphate buffered saline (PBS) for 30 min at room temperature (RT). The treated sections were then incubated either with PAM-1 (10 µg/ml) or unrelated human control IgM for 2.5 hrs. at 37°C in a humidified incubator. PSA (diluted 1:100) for prostate tissue and CAM-Keratin (BD Biosciences, Erembodegem-Aalst, Belgium, diluted 1:50) for breast tissue served as positive controls. After incubation the sections were washed three times with Tris/NaCl, followed by incubation with peroxidase-labelled rabbit anti-human or rabbit anti-mouse conjugate (Dako, Hamburg, Germany) diluted 1:50 in PBS containing rabbit serum (for antibody PAM-1) for 1 h at RT. After washing three times with Tris/NaCl and incubation in PBS for 10 min staining was performed with diaminobenzidine (0.05%)-hydrogen peroxide (0.02%) for 10 min at RT. The reaction was stopped under running tap water, and sections were counterstained with hematoxylin. After mounting with glycerol gelatin the sections were analyzed using light microscopy.

2.3. Flow cytometry

The cell lines BPH-1 (benign prostate hyperplasia) and DU-145 (prostate adenocarcinoma) were used for the analysis of CFR-1/PAM-1 receptor expression on prostate cells. Cells were grown to subconfluency in complete medium, detached with Trypsin/EDTA and incubated on ice for 1 h for recreation. Then cells were subsequently incubated on ice with PAM-1 at a final concentration of 40 µg/ml and human isotype-matched control antibody (Chrompure human IgM, Dianova, Hamburg, Germany) at the same concentration for 15 min. This was followed by incubation with a FITC-labeled rabbit anti-human IgM antibody (Dianova) for 15 min on ice. Antibodies were optimally diluted in PBS containing 0.01% sodium azide. Cells were analyzed by flow cytometry (FACScan; Becton Dickinson, USA).

3. Results

3.1. Expression of CFR-1/PAM-1 on normal and malignant prostate and breast tissue

The CFR-1/PAM-1 receptor defined by the human monoclonal antibody PAM-1 is a new membrane-bound proliferation marker, specifically expressed on malignant, but absent on non-transformed tissue. This has been shown in detail in a previous publication [11].
Fig. 1. Immunohistochemical staining of antibody PAM-1 on normal and maligne breast and prostate tissues. Paraffin sections were stained with hematoxylin-eosin, positive control (PSA for prostate tissue, CAM5.2 for breast tissue), secondary antibody alone as a negative control and antibody PAM-1: A, normal breast tissue; B, invasive ductal adenocarcinoma of the breast; C, normal prostate tissue; D, adenocarcinoma of the prostate. Original magnification, x100.

However, to confirm and to illustrate the absence of the CFR-1/PAM-1 receptor from normal prostate and breast tissue, selected paraffin tissue sections were stained with antibody PAM-1. On normal tissue sections of the prostate and the breast (Fig. 1A and C) no expression of CFR-1/PAM-1 could be detected. In contrast a specific staining was observed on carcinoma cells (Fig. 1B and D; Table 1). These data demonstrate that the CFR-1/PAM-1 receptor is expressed specifically on malignant prostate and breast tissue.

3.2. Expression of CFR-1/PAM-1 on premalignant and malignant breast tissue

Breast cancer is the most common malignancy affecting women in Europe and North America and is the second leading cause of cancer death in women after lung cancer [14]. The most potent precursor of invasive carcinoma, the intraepithelial (noninvasive) neoplasia of breast comprises two types: lobular carcinoma in situ and ductal carcinoma in situ [15]. The early diagnosis of breast cancer in its pre-invasive phase is important for prognosis and therapy efficacy.

To study and demonstrate the highly specific expression of CFR-1/PAM-1 on premalignant and malignant breast tissue 42 different cases with varying malignancy stages were tested immunohistochemically. The staining results, patient data and tissue classifications are summarized in Table 1. As shown in Fig. 2, breast cancer precursor lesions D-CIS (ductal carcinoma in situ) (Fig. 2 A, B) and L-CIS (lobular carcinoma in situ) (Fig. 2C, D) showed a broad, intensive and homogeneous staining with antibody PAM-1 on all tested tissue samples. Every tested precancerous or cancerous breast tissue sample showed a specific expression of CFR-1/PAM-1 on aberrant cells.

3.3. Expression of CFR-1/PAM-1 on premalignant and malignant prostate tissue

Prostate cancer is the most common cancer diagnosed in men older than 40 years of age. Approximately 189,000 men were diagnosed with prostate cancer in 2002, accounting for approximately 30% of all new cancers diagnosed in men. However, the mortality rates have been declining by an average of 4.5% annually since 1994 [16].
Table 1
Expression of CFR-I/PAM-1 on prostate and breast precancerous stages and carcinomas

<table>
<thead>
<tr>
<th>Organ</th>
<th>Grade</th>
<th>Stage</th>
<th>Age</th>
<th>PAM-1 +/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>D-CIS</td>
<td>Low grade</td>
<td>61–77</td>
<td>3/0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High grade</td>
<td>47–75</td>
<td>11/0</td>
</tr>
<tr>
<td>L-CIS</td>
<td></td>
<td>High grade</td>
<td>48–50</td>
<td>5/0</td>
</tr>
<tr>
<td>Invasive ductal Ca.</td>
<td>1–3</td>
<td>pT1b pN0 – pT2c pN1b</td>
<td>37–88</td>
<td>16/0</td>
</tr>
<tr>
<td>Invasive lobular Ca.</td>
<td>2–3</td>
<td>pT1b pN0 – pT3 pN0</td>
<td>40–89</td>
<td>7/0</td>
</tr>
<tr>
<td>Prostate</td>
<td>PIN</td>
<td>Low grade</td>
<td>56–74</td>
<td>7/0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High grade</td>
<td>55–72</td>
<td>8/1</td>
</tr>
<tr>
<td>Adeno-Ca.</td>
<td>GIIa-GIIIb</td>
<td>pT2b pN0 – pT3b pN1</td>
<td>57–78</td>
<td>14/1</td>
</tr>
</tbody>
</table>

Fig. 2. Immunohistochemical staining of PAM-1 on different precursor lesions of breast carcinomas. Paraffin sections were stained with hematoxylin-eosin, positive control (CAM5.2), secondary antibody alone as a negative control and antibody PAM-1: A, and B, ductal carcinoma in situ (D-CIS); C and D, lobular carcinoma in situ (L-CIS). Original magnification, ×100.

Diagnosis of prostate precursors is often difficult to perform for the pathologists because sometimes prostate cells do not appear cancerous, but they are not quite normal, either. These results are often reported as “suspicious”. They generally fall into 2 categories – either atypical or prostatic intraepithelial neoplasia (PIN). PIN is often divided into low grade and high grade. The importance of low-grade PIN in relation to prostate cancer is still unclear but a high grade PIN is a recognised precursor stage of prostate cancer [17].

The expression of CFR-I/PAM-1 on premalignant and malignant prostate tissue was investigated in 31 cases with different stages of malignancy by using immunohistochemistry. The staining results, patient data and tissue classifications are summarized in Table 1. All but one premalignant prostate tissues of low and high grade PIN showed CFR-I/PAM-1 expression. In Fig. 3A the expression on PIN versus on normal prostate tissue could be compared directly in one sample. While no recognizable expression of CFR-I/PAM-1 could be found on normal prostate cells, a clear staining could be found in the PIN area. In addition a clear discrimination between benign prostate hyperplasia (BPH) and PIN was possible (Fig. 3B). BPH showed no expression of CFR-I/PAM-1 while PIN showed a strong staining with antibody PAM-1. This impressive expression is also present in high grade PIN and prostate adenocarcinoma (Fig. 3C, D). In general, antibody PAM-1 shows a clear positive and homogeneous staining on nearly all tested different precursors and in addition an increasing level of CFR-I/PAM-1 expression with the grade of malignancy. In comparison, PSA
3.4. Flow cytometry

To support the immunohistochemical staining data we performed a FACS analysis on benign and malignant prostate cells. The cell lines BPH-1 (benign prostate hyperplasia) and DU-145 (prostate adenocarcinoma) were incubated with antibody PAM-1 or unrelated human isotype control to reveal the expression of CFR-1/PAM-1. Benign prostate cells do not express the CFR-1/PAM-1 receptor (Fig. 4B), while the receptor is clearly expressed on prostate carcinoma cells (Fig. 4A). These findings confirm the results obtained with im-

is not found in all carcinomas, it shows only a weak expression in most cases and in contrast to PAM-1, it is distributed inhomogeneously (Fig. 3).

Fig. 4. Immunohistochemical staining of PAM-1 on different precursor lesions of prostate carcinomas. Paraffin sections were stained with hematoxylin-eosin, positive control (PSA), secondary antibody alone as a negative control and antibody PAM-1: A, normal prostate tissue and prostate intraepithelial neoplasia (PIN); B, benign prostate hyperplasia and PIN; C, PIN; D, prostate adenocarcinoma and PIN. Original magnification, ×100.

Fig. 4. Evaluation of CFR-1/PAM-1 expression on prostate cells by FACS analysis. Benign prostate hyperplasia cells (BPH-1) and prostate carcinoma cell line DU-145 were incubated with antibody PAM-1 or isotype matched control antibody followed by treatment with FITC labeled secondary antibody. FACS analysis show a clear expression of CFR-1/PAM-1 on prostate carcinoma cells (A) but not on benign prostate hyperplasia cells (B).
munohistochemistry. CFR-1/PAM-1 is expressed exclusively on premalignant and malignant cells but not on benign ones.

4. Discussion

The fully human germline-coded monoclonal IgM antibody PAM-1 was isolated from a patient with a stomach carcinoma using the human hybridoma technology. The receptor for PAM-1 was purified from tumor cell membrane extracts and was found to be a 130 kDa integral membrane glycoprotein [8], homologous to CFR-1 [18].

In this study we showed that CFR-1, detected by the human antibody PAM-1 is, in contrast to other proliferation markers, a reliable target for the detection of precancerous and cancerous lesions. We presented new data on the unique expression of this new CFR-1/PAM-1 receptor on breast cancer precursors (D-/L-CIS) as well as on prostate precancerous lesions (PIN), demonstrating that the PAM-1 antibody is an ideal reliable diagnostic (and therapeutic) tool for the detection of precancerous and cancerous lesions of prostate and breast.

Tumor prevention, in addition to the identification of risk factors, is mainly based on the detection of cancer precursor lesions with the chance of most solid cancers being successfully treated if diagnosed early. The detection of these precancerous cells is based on a microscopic analysis of biopsy material. This morphological diagnosis, the differentiation between healthy and malignant tissue, is usually difficult to perform because the cellular changes are often minimal. Standard histological methods are also hampered by the fact that the analysis is influenced by the subjectivity of the observer [19,20]. Therefore additional immunohistochemical methods are helpful for the detection of cellular malignant changes, like, for example, the use of proliferation markers. However, most proliferation markers, like Ki67 or PCNA, do not differentiate between healthy and malignant cells, neither do they react with all tumor cells [21–23], which makes clear grading sometimes difficult.

The best way to define tumor-specific markers is via human antibodies isolated from cancer patients. Using the human hybridoma technology we have isolated and previously described a series of human monoclonal IgM antibodies, which were isolated from cancer patients and from healthy donors [10,24–26].

The human antibody SC-1 was generated from a patient with a signet-ring-cell carcinoma of the stomach [24]. The receptor for SC-1 was found to be a modified version of CD55 (DAF) [7]. Stomach carcinoma cells express two different forms of CD55/DAF on the cell surface: 1) the normal 70 kDa isoform which protects against complement attack, and 2) the recently described CD55/SC-1 apoptosis receptor [9]. The CD55/SC-1 isoform is overexpressed on gastric carcinoma cells [7,9] and has a molecular weight of approximately 82 kDa. The IgM antibody SC-1 induces apoptosis of gastric cancer cells in vitro and in vivo and is being used successfully in clinical trials [6,7,9,27–29].

Another human monoclonal antibody, called SAM-6 induces tumor-specific lipid accumulation, which leads to lipoptosis [26]. All these tumor-specific IgMs belong to the inherited pool of natural antibodies. These IgM antibodies are low affine, germ-line coded and detect specific patterns of antigens, mostly repetitive structures or carbohydrates, and remove aberrant cells by inducing apoptosis [7,8,10,12,25,26,30,31]. They are responsible for the early detection and elimination of modified, secreted, self-cells and-structures, like DNA, RNA, filaments, oxidized molecules and transformed cells. In addition they are involved in the detection and elimination of foreign particles, like bacteria, fungi and viruses [31–37].

About 30% of the circulating immunoglobulins are germline-coded natural IgM antibodies which are produced by CD5+ lymphocytes. This level can increase during certain bacterial infections, and in the case of IgM deficiencies the incidence of malignant neoplasias or infections can increase. The natural IgMs are also called autoantibodies, because they react with any kind of self-structures. But most of these autoantibodies are not pathogenic [38]. Interestingly, nearly all harmful and dangerous autoantibodies are affinity matured IgG [39,40], implicating that the failure of autoreactivity is not based on the primary recognition by the IgM, but instead on a failure of T-cell recognition and a misleading immune response. If T-cells recognize a presented self as nonself, a harmful autoimmunity is initiated.

For a long time the physiological role of this armada of IgM antibodies was not clear. Several hypotheses were made to explain the existence of this big pool of not or only slightly mutated low affine, cross-reacting and pentameric antibodies. However we think it is likely that this armada of natural IgM antibodies is the primary fighter against anything changed, accidentally
secreted or modified “self” and foreign invaders. By using this huge pool of IgM antibodies, very efficient tests can be established for the detection and elimination of precancerous and cancerous lesions.

References


