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Expression of MAGE antigens and analysis of the inflammatory T-cell infiltrate in human seminoma

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Abstract The MAGE gene family encodes antigens that are recognized by cytotoxic T-cells. The expression of MAGE antigens has been linked to tumor stage, and MAGE peptides are under investigation as possible vaccines. Seminomas are tumors that are typically accompanied by a heavy inflammatory infiltrate, but have not been studied with regard to their MAGE antigen expression and its correlation with the inflammatory infiltrate. We investigated, therefore, MAGE protein expression, the amount of cytotoxic T-cells, clonality of the lymphocytic infiltrate, apoptotic activity and occurrence of necrosis. Specimens of 27 patients with classical seminoma were examined by immunohistochemistry for CD4, CD8, CD56, CD45R0, β_2-microglobulin and HLA-DR. MAGE expression was detected with the monoclonal antibody 57B, reactive with MAGE-1, -3, -4, -6 and -12. Clonality of the inflammatory infiltrate was examined by multiplex polymerase chain reaction (PCR) analysis of the T-cell receptor rearrangement. Apoptotic cells were detected by DNA nick-end labeling of fragmented DNA, and the apoptotic index was determined semi-quantitatively. Expression of 57B was found in 19 (70%) of 27 seminomas. In all cases, more than 70% of T-cells expressed CD45R0. In four cases, a predominant infiltration of CD8-positive cytotoxic T-cells (CD4/CD8 ratio <1) was present. However, 15 seminomas showed a CD4/CD8 ratio >1. In all cases, infiltration of CD56-positive natural killer cells was only focal. HLA-DR expression was not detectable in tumor tissue; β_2-microglobulin was only focal in three cases. Analysis of the T-cell clonality revealed a polyclonal population. The apoptotic index was not significantly different in 57B-positive seminomas (4.15%) compared with 57B negative seminomas (3.80%). Also, no correlation between the 57B expression and the occurrence of necrosis was found. MAGE antigens are homogeneously expressed in most seminomas, but their presence does not appear to represent a dominant epitope responsible for the lymphocytic infiltrate.

Key words MAGE · Seminoma · Tumor-infiltrating lymphocytes

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Introduction

Malignant neoplasms often are associated with an inflammatory infiltrate, and the interaction of tumor-infiltrating lymphocytes (TIL) with the tumor tissue is believed to have an important role in some malignant neoplasms. However, for most tumors it remains controversial whether the amount of TIL is of predictive value. For certain solid tumors, a high number of TIL is associated with a better prognosis [8, 27, 33, 35, 39]. In seminomas, the presence of TIL is correlated with an improved prognosis [11, 26]. Earlier studies showed that TIL in seminomas consist predominantly of T-lymphocytes, whereas B-cells are less abundant [1, 28]. The biology of TIL migration into the tumor tissue is not yet understood. Although the presence of TIL is proposed to demonstrate an immunologic response to the tumor tissue [16], specific antigens eliciting this inflammatory

infiltration have not yet been characterized. Recent studies of T-cell receptor (TCR) α and β variable gene expression have demonstrated that TIL are clonally expanded in some tumors, suggesting an immunologic response to a particular tumor-associated antigen [30, 32, 40].

Recently, several genes or gene families encoding tumor-associated antigens have been isolated by analyzing autologous serologic or cytotoxic T-cell responses [2, 6, 36, 37]. Cancer/Testis ('CT') antigens, a subgroup of these tumor-associated antigens, are expressed in a variety of malignant neoplasms, and in the testis as the only normal tissue [36]. The MAGE gene family was the first CT antigen isolated [37], and 17 different MAGE encoding genes have now been identified, of which the MAGE-1, -2, -3, -4, -6 and -12 genes show the typical CT expression pattern [10, 23, 24]. Current knowledge of the expression pattern of most CT antigens is mainly based on mRNA typing, and only recently have analyses of their protein expression become available [17]. The monoclonal antibody (mAb) 57B was generated against the MAGE-3 recombinant protein and originally thought to be specific for the MAGE-3 antigen using immunoblotting analysis [15, 21]. While 57B's fine specificity, as to which MAGE antigen is primarily detected, is still under debate [17, 18, 22], it resembles one of the few MAGE-reactive mAbs useful for immunohistochemical analyses of MAGE antigen expression.

While MAGE gene expression has been analyzed in a broad spectrum of tumors [19], surprisingly little is known about the presence of MAGE genes in seminomas. Since normal testicular germ cells consistently express MAGE antigens, an analysis of their malignant form is a logical consequence. A preliminary analysis by our group did in fact indicate a high expression of MAGE antigens in this type of neoplasm [17].

A typical morphological feature of seminomas, the intense lymphocytic infiltrate, might link tumor-associated antigens to components of the inflammatory response. Analyses of the MAGE protein expression and its correlation with TIL in human seminomas have not been performed. Therefore, the purpose of this study was to investigate the expression of the MAGE antigens in seminomas and to correlate the MAGE antigen expression with the type and number of T-cells and the clonality of the lymphocytic infiltrate. In addition, the apoptotic index and the occurrence of necrosis within the tumor were determined.

Material and methods

Patients and specimens

Formalin-fixed, paraffin-embedded semi-castration specimens of 27 patients with classical seminoma were studied. All patients underwent surgery between 1992 and 1995 at the Department of Urology, University Hospital Mannheim, Germany. The mean age was 38 $\pm$ 11 years (range 23–63 years). Preoperative serum β-human chorionic gonadotropin (β-HCG) levels (normal range < 5 IU/l for male patients) were determined in 26 patients. The mean serum level was 74.0 $\pm$ 129.8 IU/l for β-HCG-positive ($n = 7$) and 1.46 $\pm$ 0.70 IU/l for β-HCG-negative patients ($n = 19$). Histologic evaluation of the tumor stage and assessment of necrosis was performed by conventional light microscopy.

Antibodies

The monoclonal antibody 57B was described earlier [21]. A concentration of 1.0 µg/ml was used. The T-cell infiltrate was characterized using monoclonal mouse antibodies against CD4, CD8, CD56 (Loxo, Dossenheim, Germany) and CD45R0 (Dako, Hamburg, Germany) at concentrations of 1:20 for CD4 and CD8 and 1:100 for CD45R0 and CD56. HLA class I was evaluated using an mAb against β_2-microgobulin (Dako; 1:1000). HLA class II expression was studied with an anti-HLA II mAb (Loxo; 1:50). A heat-based antigen retrieval method was performed using a steamer (30 min) for 57B and a microwave oven (3 $\times$ 5 min) for CD4, CD8 and CD56. Citrate buffer (10 mM, pH 6.0) served as the retrieval solution for all antibodies. Pre-treatment with pronase was performed for CD45R0, β_2-microgobulin and HLA II.

Immunohistochemistry

Serial sections, 2 µm thick, were cut in a SM200R microtome (Leica, Bensheim, Germany) and mounted on silane-coated glass slides. Sections were dried in an incubation chamber at 37 °C and deparaffinized in xylene and a series of graded alcohols. For detection of CD4, sections were incubated with the primary antibody at 4 °C overnight; for CD8, CD45R0, CD56, sections were incubated with β_2-microgobulin and HLA II for 1 h at room temperature in a dark humid incubation chamber. After washing in Tris-buffered saline (TBS, pH 7.60), the secondary biotinylated goat anti-mouse antibody (Dianova, Hamburg, Germany) was applied for 30 min. The streptavidin–alkaline phosphatase conjugated complex (Dianova) was added for 30 min after washing in TBS. The use of the substrate naphthol with the chromogen Fast Red (Boehringer Mannheim, Germany) resulted in a red amorphous precipitate at the binding site. Slides were mounted in Kaiser's glycerin gelatin (Merck, Darmstadt, Germany).

For 57B, incubations were performed at 4 °C overnight. The detection of the primary was performed using a biotinylated horse anti-mouse antibody followed by an avidin–biotin–peroxidase method (ABC-Elite kit; Vector Laboratories, Burlingame, Calif., USA). DAB (3,3′-diaminobenzidinetetrahydrochloride; BioGenex, San Ramon, Calif., USA) was used as chromogen. Negative controls were incubated with buffer instead of the primary antibody. All sections were counterstained in Mayer's hematoxylin (Merck).

The CD4/CD8 ratio was determined by counting positive cells in three corresponding high-power fields of serial sections. Tumors were considered positive when more than 20% of tumor cells were 57B-reactive.

Multiplex polymerase chain reaction analysis of the TCR γ-chain rearrangement

Experiments were performed as described previously [12, 38]. Genomic DNA was extracted from paraffin-embedded tissue using standard protocols [31]. The multiplex polymerase chain reaction (PCR) contained 11 primers: eight were specific for variable genes in the V region, three for joining segments in the J region of the TCR γ-chain [12]. For the PCR, 1.0 µg of genomic DNA was used in a 50-µl PCR reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8% bovine serum albumin (BSA), 1.2 U Taq polymerase (Boehringer Mannheim), 1.0 pmol of V-primer and 1.5 pmol of J-primer. Thermocycling conditions were as follows: initial denaturation step at 94 °C for 7 min, 45 cycles at 94 °C (1 min), 62 °C (1 min), 72 °C (1 min), terminal extension of 20 min at 72 °C. Five microliters of the PCR amplification product was loaded on a 7.5% polyacryla-

mide gel and stained with a 0.3% $AgNO_3$ solution. A probe of a cutaneous T-cell lymphoma was used as a positive control.

Nick-end labeling procedure

For the in situ detection of apoptotic cells, a modified technique of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick-end labeling (TUNEL) method was applied [13]. After digestion of nuclear proteins by 5 μg/ml proteinase K (Boehringer Mannheim) for 25 min, TdT (Promega, Madison, Wis., USA) at a concentration of 20 U/μl was used to insert biotin-16-dUTP (1 mM; Boehringer Mannheim) to the ends of the DNA fragments. A streptavidin–alkaline phosphatase conjugated complex (Dianova), naphthol and Fast Red (Boehringer Mannheim) served as a detection system. After washings in phosphate-buffered saline (PBS; pH 7.40), sections were counterstained with Mayer's hematoxylin (Merck).

The apoptotic index was determined by counting the number of apoptotic tumor cells within at least 1000 tumor cells. Proof of significance was performed using the Student's *t*-test or Wilcoxon rank sum test.

Results

57B reactivity and correlation with clinical data

57B immunoreactivity was present in non-neoplastic spermatogenic cells within preserved seminiferous tubules and followed the pattern previously described [7, 17]. Germ cells of late-stage spermatogenesis, such as spermatids, non-spermatogenic cells, e.g. Sertoli or Leydig cells, or any other non-tumorous tissue component, remained negative (Fig. 1). 57B reactivity could be found in 19 (70%) of 27 seminomas (Fig. 2). In 12 cases, more than 50% of the tumor was immunoreactive. In seven cases, 20–50% of tumor cells were positive. MAGE protein was localized predominantly in the cytoplasm and in the nucleus to a variable degree. In 57B-positive patients ($n = 19$), the β-HCG serum level was 24.75 ± 83.5 IU/l and in 57B-negative patients ($n = 7$), it was 10.77 ± 15.8 IU/l, showing no statistically significant difference ($P = 0.06$). Of the 21 stage pT1 seminomas, 17 exhibited 57B reactivity and four remained negative. Among the six cases of pT2 seminoma, only two cases showed 57B reactivity.

Analysis of the T-cell infiltrate and 57B reactivity

In all 27 cases, more than 70% of the lymphocytic infiltrate was positive for CD45R0. In 23 seminomas, the infiltration consisted of more CD4-positive than CD8-positive T-cells (CD4/CD8 ratio > 1), of which 15 were 57B-positive and eight were 57B-negative. Four cases revealed a CD4/CD8 ratio < 1, all of which were 57B-positive. Tumor infiltration by CD56-positive natural killer cells was only focal and represented only a minority of cytotoxic cells, which appeared independent of MAGE protein expression. Expression of HLA II was restricted to the lymphocytes; no expression was found in tumor tissue. β_2-microgobulin was also present in the majority of T-cells. In three cases, a focal expression of β_2-microgobulin was present in tumor cells.

TCR analysis of the γ-chain rearrangement revealed no distinct band in any case, indicating the presence of polyclonal T-cell populations (Fig. 3, lane 1–8). The positive control of a cutaneous T-cell lymphoma showed a distinct band, indicating a monoclonal γ-chain rearrangement, typical for this neoplasm (Fig. 3, lane P).

Necrosis, apoptotic index and 57B reactivity

Necrosis was found in eight cases, yet absent in 19 seminomas. In six of eight cases with necrosis, the tumor was found to be 57B-positive, while two remained

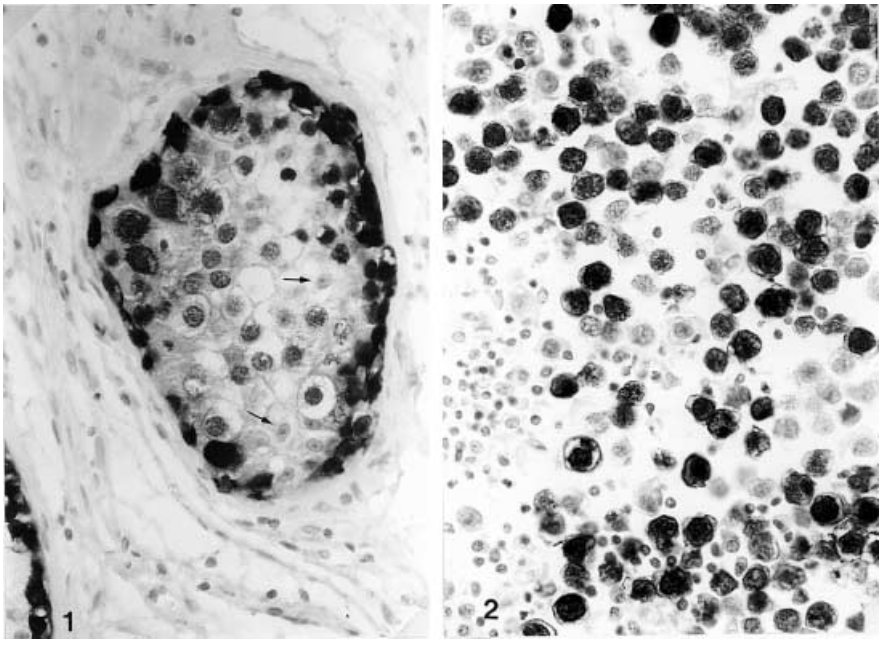

Fig. 1 Expression of 57B, reactive to MAGE-1, -3, -4, -6 and -12 proteins, in normal testicular tubules. Note expression in all cells of spermatogenesis but not in Sertoli cells (*arrows*). Magnification ×100

Fig. 2 Classical seminoma strongly reactive to the monoclonal antibody 57B, representing a MAGE-1, -3, -4, -6 and -12 protein expression. Magnification ×100

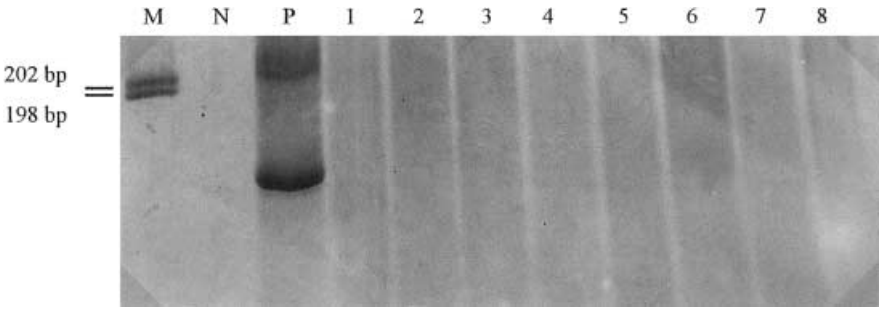

Fig. 3 T-cell receptor analysis of the γ-chain rearrangement: *M* marker, *N* negative control, *P* positive control (cutaneous T-cell lymphoma). A distinct band at 180 bp indicates the monoclonal T-cell population. No distinct band is detectable in seminoma probes (*lanes 1–8*), indicating a polyclonal T-cell population

immunonegative. However, 13 of 19 non-necrotic seminomas were 57B-positive, while the remaining 6 were 57B-negative. Hence, there appeared to be no correlation between MAGE protein expression and the presence of necrosis.

In all cases, apoptotic tumor cells were found mainly in the vicinity of TIL. The mean apoptotic index for all seminomas was 4.05 ± 2.37%. The difference in the apoptotic index between 57B-positive seminomas (4.15 ± 2.58%) and 57B-negative seminomas (3.80 ± 1.89%) was not statistically significant ($P = 0.36$; Fig. 4).

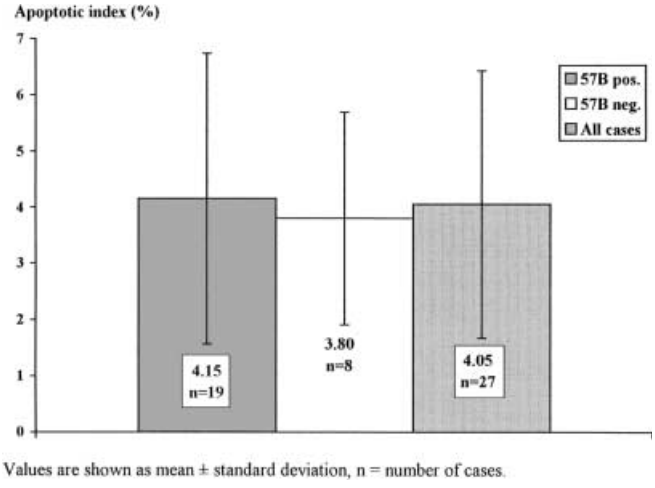

Fig. 4 Apoptotic index and 57B reactivity in human seminomas

Discussion

Classical seminomas are characterized by an abundant lymphocytic infiltrate, which is regarded as an immunologic response to the tumor tissue resulting in a favorable clinical outcome [11, 26, 35]. As shown in previous studies, the inflammatory infiltrate consists mainly of T-cells [1, 28]. Specific tumor antigens associated with this particular inflammatory reaction have not yet been identified. MAGE antigens are expressed in a wide variety of malignant neoplasms but are not expressed in normal tissue, except testis [19]. Tumors of various histologic types, such as melanomas, several carcinomas and some sarcomas, express MAGE antigens [36]. Seminomas represent a malignant germ cell tumor derived from intratubular germ cell neoplasia (ITGCN) [5]. Two models for the origin of ITGCN exist: (a) that it develops from polyploidization of the transformed gonocyte, followed by DNA loss and i(12p) formation [9]; (b) that the target cell is represented by the meiotic pachytene spermatocyte expressing wild-type p53 [5]. In this and previous studies [7], MAGE antigen expression could be demonstrated in spermatogonia as well as spermatocytes, as the only cell types regularly expressing MAGE antigens in normal tissues. In seminomas, about 70% of cases showed a MAGE mRNA expression [14]. An immunohistochemical analysis by our group revealed homogeneous immunoreactivity in 7 of 10 seminomas with mAb 57B in frozen seminoma samples [17]. Another study found 10 of 24 seminomas positive for 57B staining [7]. However, no study attempted to analyze the correlation of MAGE antigen and/or mRNA expression and the components of the accompanying inflammatory reaction.

In the present study we confirm the presence of MAGE antigen, as detected by the mAb 57B, in the majority of seminomas. Though MAGE antigens are expressed in germ cells of the normal testis, the lack of MHC class I on germ cells (making the testis immunoprivileged) prevents the recognition of these antigens by cytotoxic T-cells [25]. While MAGE antigens are not expressed in normal non-testicular tissues, they are present in some of their malignant tumors. In seminomas, however, an inverse situation exists: though MAGE antigens are generally expressed in normal spermatogenic cells, surprisingly not all seminomas show a MAGE antigen expression. In our study, 57B-reactivity was present in 70% of cases; hence, the MAGE-antigen is lost in 30% of tumors during carcinogenesis. Although the function of MAGE antigens is still unclear, our study suggests that MAGE antigens may play a different role during tumorigenesis in testis than in other tumor types. During tumor development, the immunoprivilege is lost and immunocompetent cells may invade. Although cytotoxic T-cells are abundant in seminomas, no correlation between the 57B reactivity and the intensity and the composition of the inflammatory infiltrate could be found in our series. Also, no

correlation with apoptotic activity or with occurring necrosis was demonstrable. Further studies on MAGE expression in ITGCN may give a clue to the role of MAGE antigens in the tumorigenesis of seminoma.

MAGE proteins encode peptide antigens that are presented in association with HLA class I molecules and are recognized by cytotoxic T-cells [19]. In normal testicular tubules, HLA class I is not present in germ cells or Sertoli cells [3]. In ITGCN, however, HLA class I is present in Sertoli cells but not in neoplastic cells [3, 4]. This finding suggests that the cytotoxic inflammatory infiltrate in seminoma may be caused by the recognition of HLA class I antigens of Sertoli cells and not of tumor cells when the basement membrane is disrupted during tumor invasion [3]. Studies on HLA class I expression in seminomas have yielded discrepant results. Whereas some investigators found no HLA class I expression [1, 3, 29], others reported an expression in a high proportion of seminomas [20, 34]. The expression of HLA class I in at least some seminomas may, among other mechanisms, be the result of a gene deactivation during the multi-step process of tumorigenesis. Since Sertoli cells can only rarely be found in seminomas, additional mechanisms might be responsible for the induction of a dense lymphocytic infiltration within the tumor. In our series, HLA class I, as represented by the expression of β_2-microglobulin, was present only focally in three seminomas (whereas T-cells exhibited a strong β_2-microglobulin expression in all cases). One reason for the lacking correlation of the intensity and/or quality of the lymphocytic infiltrate with the MAGE expression in tumor cells could be the absence of HLA class I expression. Since the 57B reactivity correlates neither with the amount and composition of the inflammatory infiltrate nor with the apoptotic index and the occurrence of necrosis, the MAGE antigens as detected by 57B do not appear to be dominant epitopes for the lymphocytic infiltrate in seminoma. Our study suggests that in seminomas, despite their expression of MAGE antigen in a high proportion of cases, additional factors should be considered for immunologic therapeutic approaches.

Further studies on MAGE expression in testicular tumors and ITGCN are needed to elucidate the possible role of MAGE antigens in the tumorigenesis of seminoma.

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