MAGE, BAGE and GAGE: tumour antigen expression in benign and malignant ovarian tissue

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Summary To determine if ovarian cancer patients would be suitable for MAGE-peptide vaccine-based immunotherapy, the frequency of expression of the MAGE-1–4 genes in ovarian tumours was assessed using reverse transcription polymerase chain reaction (RT-PCR) and product verification with digoxigenin-labelled oligonucleotide probes specific for each MAGE gene. In addition, the frequency of expression of more recently discovered tumour antigens (BAGE, GAGE -1, -2 and GAGE -3, -6) was established using RT-PCR and ethidium bromide staining. In this study 1/16 normal ovarian tissue specimens and 11/25 benign lesions expressed MAGE-1. In non-malignant tissue there was preferential expression of MAGE-1 in premenopausal women. A total of 15/27 malignant specimens expressed MAGE-1, including 10/14 serous cystadenocarcinomas. Expression of other tumour antigens was infrequent. The finding of MAGE-1 expression in both benign and malignant tissue questions previous assumptions regarding the role of MAGE genes in carcinogenesis. In addition, preferential MAGE-1 gene expression in non-malignant premenopausal tissue suggests that the MAGE genes may be involved in cellular proliferation as opposed to carcinogenesis or possibly that MAGE gene expression is under cyclical hormonal control. Finally, this study indicates that serous cystadenocarcinomas may be suitable tumours for MAGE-1 peptide immunotherapy.

Keywords: MAGE; BAGE; GAGE; ovarian; tumour antigen; cancer immunotherapy

Ovarian cancer is the most common cancer of the female genital tract in the developed world and in the UK is responsible for approximately 6% of all female cancer deaths (Office of Population Censuses and Surveys, 1993). The prognosis of women diagnosed with this condition is generally poor with an overall 5-year survival of only 30%. The survival is much greater for early ovarian cancer (stage 1, 5-year survival 79%; Petterson, 1990), and the development of a screening test to detect early malignancy is seen as a priority by many investigators. However, the natural history of this disease is poorly understood and it may be that the disease does not have the characteristics that make it suitable for screening (Hulka, 1988). If the tumour has spread by the time of diagnosis a surgical cure is unobtainable. However, primary cytoreduction and intervention cytoreduction are now accepted standards of care (Hacker et al., 1983; Van der Burg et al., 1995), and subsequent treatment is usually in the form of chemotherapy. A large number of chemotherapeutic agents has been shown to be active in epithelial ovarian carcinoma, including alkylating agents, cytostatic antibiotics, platinum compounds, taxanes and topoisomerase modifiers. The fact that so many agents have a role in the management of this disease is self-evidently a reflection that none is entirely efficacious or appropriate for use in all circumstances. New treatment modalities are needed before a significant improvement can be expected in the prognosis of women diagnosed with this condition.

One such potential new therapy is antigen-specific immunotherapy with MAGE gene products. Since the discovery of the MAGE-1 antigen (Van Der Bruggen et al., 1991) this area of research has progressed rapidly. The MAGE gene family comprises a series of 12 closely related genes (De Plaen et al., 1994). Of these, MAGE-1, -2, -3, -4, -6 and -12 have been shown to be expressed in a variety of tumours of different histological type (Brasseur et al., 1992; Weynants et al., 1994; Inoue et al., 1995; Patard et al., 1995). MAGE-1 and MAGE-3 are targets for specific immunotherapy as they encode peptide antigens that are presented in association with HLA class I molecules and are recognized by cytotoxic T lymphocytes (CTLs). Clinical trials have been initiated to evaluate the role of these peptides as ‘tumour vaccines’, designed to break tolerance that may exist to these antigens and potentiate CTL activity.

MAGE gene expression in malignant ovarian tumours has previously been described (Yamada et al., 1995). We now report our own findings in malignant tumours and in addition describe MAGE gene expression in a range of benign ovarian pathological tissue. We also report the frequency of expression of other ‘tumour antigens’ – BAGE, GAGE-1 and -2 and GAGE-3 and -6 (Boel et al., 1995; Van Den Eynde et al., 1995) – which may have a future therapeutic role. Our findings indicate a potential for expanding the MAGE peptide vaccine programme to include some forms of ovarian tumours, while questioning previous assumptions regarding the role of the MAGE gene family in carcinogenesis.

MATERIAL AND METHODS

Tissue sample collection

Tissue for this study was collected from women undergoing surgical management of gynaecological conditions at Derby City General Hospital, Derby, Jessop Hospital for Women, Sheffield, and Northern General Hospital, Sheffield, UK. Samples were collected at the time of surgical excision and snap frozen in the vapour phase of liquid nitrogen. All tissue was subsequently stored in liquid nitrogen until laboratory processing.
RNA extraction and cDNA synthesis

Total RNA was isolated from the frozen tissues using the RNAzol method according to the manufacturer’s guidelines (Biotec, Houston, TX, USA). For cDNA synthesis, 2 μg of total RNA was prepared in diethyl-pyrocarbonate-treated water to a volume of 9.5 μl and mixed with 0.5 μl of oligo-(dT)_{12-18} at 0.5 μg μl⁻¹ (Pharmacia Biotech, St Albans, UK), 0.5 μl of RNaseguard at 31 600 units ml⁻¹ (Pharmacia), 4.0 μl of 5 x first-strand buffer (Life Technologies, Paisley, UK), 2.0 μl of 0.1 M DTT, 2 μl of each dNTP at 10 mM, 1.0 μl of Superscript reverse transcriptase at 200 U μl⁻¹ (Life Technologies) to a total volume of 20 μl and incubated at room temperature for 10 min and then 44°C for 2 h. Following incubation the cDNA was diluted to 10 μl with water and stored at −20°C.

PCR amplification

The integrity of the RNA was confirmed by performing PCR amplification of the cDNA with primers for porphobilinogen deaminase (PBGD) (Finke et al, 1993). The presence of cDNA for MAGE-1 -2 -3 and 4 was then determined by PCR amplification in a 50-μl reaction containing 5.0 μl of cDNA, 5 μl of 10 x PCR buffer (Boehringer Mannheim UK, Lewes, UK), 0.1 μl of each dNTP at 10 mM, 0.5 μl of each primer at 80 μm (see below), 1.0 unit of Taq polymerase (Boehringer) and 38.4 μl of water. The presence of cDNA for BAGE, GAGE-1, -2 and GAGE-3, -6, was determined by similar PCR amplification reactions – on these occasions however using 38.1 μl of water and 1 unit of DNA polymerase (Primezyme, Biometa). The reaction mixtures were then subjected to the appropriate PCR programmes as described in Table 1.

Oligonucleotide primers for PCR amplification

The oligonucleotide primers used were specific for each gene. All primers corresponded to sequences located in different exons in order to prevent false positives caused by genomic DNA contaminating the RNA preparations. The primer sequences for MAGE-1, -2, -3 and 4 are described in Patard et al (1995). The other primer sequences used are described in Table 2.

Detection of PCR products

After amplification, PCR products were prepared with 50 μl of chloroform and 12.5 μl of bromophenol blue. The products were then size-fractionated in 2% agarose gels containing ethidium bromide and visualized using UV irradiation.

Further verification of the specific nature of the MAGE PCR products was obtained by probing with a digoxigenin-labelled oligonucleotide probe specific for individual MAGE genes. In brief, following size fractionation PCR products were Southern blotted onto Hybond-N nylon membranes, subjected to digoxigenin-labelled oligonucleotides (see below), processed according to manufacturer’s guidelines with the digoxigenin luminescence detection kit for nucleic acids (Boehringer Mannheim UK, Lewes, UK) and exposed to reflection autoradiography film (Dupont).

Oligonucleotide probes for Southern blotting

The synthesis and sequences of the oligonucleotide probe for each MAGE gene is described in Mulchahy et al (1996).

Control RNA samples

Control cDNA samples were included in each PCR amplification. Melanoma cell line MZ2-MEL-30 expresses MAGE-1, -2 and -3, BAGE, GAGE-1, -2 and GAGE-3, -6. RNA prepared from this cell line was therefore used as a control for expression of these genes. The sarcoma cell line LB23-SAR expresses MAGE-4, and RNA from this cell line was used as a control for MAGE-4 gene expression.

The level of MAGE, BAGE and GAGE expression in each sample was classified positive or negative. A positive result indicates a level of expression equal or greater than 1% of that in the reference cell line, i.e. MZ2-MEL-30 (MAGE-1, -2, -3, BAGE, GAGE-1, -2 and GAGE-3, -6) and LB23-SAR (MAGE-4). A negative result indicates a level of expression less than 1% of that in the reference cell line.
RESULTS

In this study a total of 74 ovarian tissue specimens were analysed for expression of MAGE-1, -2, -3, -4, BAGE, GAGE-1, -2 and GAGE-3, GAGE-3, -6 using RT-PCR amplification with oligonucleotide primers specific for each gene and detection of PCR products as detailed in the previous section. The 74 specimens comprised 16 normal ovaries, 25 benign ovarian lesions, 27 malignant ovarian lesions and six metastatic lesions from ovarian carcinoma.

An overview of the expression of each gene in this study of ovarian tissue is provided in Table 3. A total of 1/16 normal tissue specimens expressed MAGE-1 and another expressed MAGE-3. A total of 11/25 (44%) benign pathological lesions expressed MAGE-1 and one expressed MAGE-2. In total, 15/27 (56%) malignant ovarian tissue specimens expressed MAGE-1, with other gene expression in this group detailed in Table 3. Two out of six metastatic lesions expressed MAGE-1. There was no pattern of quantitative differences in the level of MAGE-1 gene expression between the malignant and non-malignant ovarian tissue specimens. Figure 1 shows representative results. The normal ovary specimen OV35 has a lower level of MAGE-1 gene expression than some of the other tissues studied. In this study there is infrequent expression of all MAGE, BAGE and GAGE genes tested in ovarian tissue apart from MAGE-1 expression in benign and malignant pathological tissue.

A detailed breakdown of the histological type of non-malignant lesions (normal and benign specimens) studied and the MAGE-1 gene expression in these lesions is shown in Table 4. It can be seen that a variety of different lesions express MAGE-1, including inclusion cysts, cystadenomas and endometrioid cysts.

In non-malignant ovarian tissue a relationship was shown between the menopausal status of the women providing the specimen and the frequency of MAGE-1 expression. A total of 10/21 (48%) samples obtained from premenopausal women expressed MAGE-1 whereas only 2/20 (10%) examples from postmenopausal women expressed this gene. This association reached statistical significance using a chi-squared test ($P < 0.05$). Note only 3/16 normal specimens came from premenopausal women and that one of these was MAGE-1 positive.

Of the malignant tissue specimens included in this study serous cystadenocarcinomas (10/14), mucinous carcinomas (2/7) and granulosa cell tumour (2/2) expressed MAGE-1 mRNA. MAGE-1 expression was also found in 1/2 Krukenberg tumours (breast primary) and 2/6 metastatic specimens. Expression of MAGE-2, -3, -4, BAGE, GAGE-1, -2 and GAGE-3, -6 was infrequent (see Table 5).

A total of 23 ovarian carcinomas of epithelial origin are included in this study. There is preferential expression of the MAGE-1 gene in serous tumours (10/14, 71%), with relatively infrequent expression in other tumours of epithelial origin (2/9, 22%). The association between serous histology and MAGE-1 expression is statistically significant (chi-squared, $P < 0.05$). Close analysis of MAGE-1 expression in serous cystadenocarcinomas reveals a trend towards expression in early stage (6/6 stage I lesions MAGE-1 positive, 4/8 stage II, III and IV lesions MAGE-1 positive).

In malignant tissue specimens studied we found no relationship between MAGE gene expression and patient age, menopausal status, preoperative CA125 and outcome (although follow-up times were insufficient to conduct a full analysis of this parameter).

DISCUSSION

Ovarian carcinoma has a poor overall prognosis, reflecting a disease that is usually diagnosed at an advanced stage and the limitations of current screening and treatment modalities. Much work is in progress to develop screening programmes that may improve survival by assisting with earlier diagnosis. Progress is also being made in improving surgical techniques and efficiency and optimizing post-operative chemotherapy regimens. In addition, new chemotherapeutic agents are continually being introduced and some offer potential for the future.

New treatment modalities may also contribute to the therapeutic armamentarium for women diagnosed with this condition. One area of research currently stimulating much interest is that of tumour immunology and immunotherapy. The use of immunotherapy in ovarian carcinoma is not new; however, previous work has been limited in effectiveness (Berek et al, 1995). A new form of antigen-specific immunotherapy has been suggested by the discovery of the MAGE gene family and related tumour antigens.
It has previously been reported that MAGE, BAGE and GAGE genes are expressed only in malignant tissue, with the exception of the male germline cells within the testis and the placenta (De Plaen et al., 1994; Takahashi et al., 1995). These findings are potentially highly significant because the gene products may represent tumour-specific targets for immunotherapy. It is known that MAGE-1 and -3 are targets for specific immunotherapy as they encode peptide antigens that are presented in association with HLA class I molecules and are recognized by CTL. MAGE-1 is expressed in association with HLA-A1 and -CW1601 (Traversari et al., 1992; Van der Bruggen et al., 1994a), whereas MAGE-3 is expressed in association with HLA-A1 and HLA-A2.01 (Gaugler et al., 1994; Van der Bruggen et al., 1994b). Pilot studies have commenced to assess the value of MAGE-1-A1, MAGE-3-A1 and MAGE-3-A2 peptides as tumour vaccines in a number of tumour types – including malignant melanoma – that have previously been shown to express the MAGE genes. It is hoped that immobilization with these peptides will induce a CTL response resulting in tumour regression. It is as yet too early to say whether this will become established as an effective form of cancer therapy. However initial reports suggest there is reason for optimism (Marchand et al., 1995).

In this study we have analysed normal ovarian tissue and a wide variety of benign and malignant ovarian pathological specimens for expression of the MAGE, BAGE and GAGE gene families. Our findings contribute significantly to the knowledge in this field of study and have implications for MAGE peptide vaccine clinical trials.

A total of 1/16 normal ovarian tissue specimens analysed expressed MAGE-1 and 1/16 expressed MAGE-3. The MAGE-1-positive normal ovary was the contralateral ovary to a MAGE-1-positive stage Ia mucinous carcinoma. The MAGE-3 positive normal ovary was the contralateral ovary to a stage IIc serous cystadenocarcinoma of unknown MAGE expression. Bilateral ovarian carcinomas are known to occur and it is therefore possible that the positivity for MAGE in these two samples reflected the heterogeneity of the tissues analysed – with some tumour cells being present in the RT-PCR samples but absent in the samples examined by the pathologist.

Our findings in benign ovarian pathological specimens were totally unexpected, with 11/25 lesions expressing MAGE-1 (Table 3). Of these benign lesions expressing MAGE-1, inclusion cysts, serous cystadenomas and mucinous cystadenomas are considered putative precursor lesions, whereas fibromas are not (Table 4). The natural history of ovarian carcinoma is poorly understood; there is no general agreement on the most likely premalignant lesion and it may be that the different histological subtypes have a different natural history. The results of this study do not show preferential MAGE gene expression in any candidate precursor lesion over any other and so unfortunately do not implicate any particular lesion.

One of the most significant observations from this study may be the preferential MAGE-1 gene expression in non-malignant ovarian tissue obtained from premenopausal women. This finding has implications for current understanding of the role of the MAGE genes. Whereas trials have rapidly been developed to exploit the therapeutic potential of MAGE gene expression, the question as to the role of MAGE remains unanswered. The finding of MAGE gene expression exclusively in malignant tissue implies a role in carcinogenesis; however, none has yet been proven. A direct relationship has been shown between MAGE gene expression and tumour progression (Brasseur et al., 1995); one might therefore anticipate that MAGE gene expression is a relatively late event in tumorigenesis and is implicated in tumour progression. However, no other evidence has been presented to support this hypothesis. Indeed it is open to question whether the MAGE genes have a specific role or whether their expression in malignant tissue is simply a consequence of the demethylation process that occurs in many cancers. A number of authors have shown that MAGE gene expression can be up-regulated by the demethylating agent 5-Aza-2'-deoxycytidine (Weber et al., 1994; De-Smet et al., 1996; Mori et al., 1996; Shichijo et al., 1996). The study of MAGE-1 protein expression with anti-MAGE-1 monoclonal antibodies could provide further information as to the role of MAGE genes. However, at present there are no reliable commercially available antibodies.

The finding of preferential MAGE-1 expression in non-malignant tissue has two possible explanations. Firstly the possibility must be raised that MAGE-1 expression is under cyclical hormonal control. However, there is no suggestion that tumours previously shown to express MAGE-1 are under such hormonal control and therefore this explanation would seem unlikely. A more acceptable explanation may be that MAGE-1 expression occurs in the ovary.

### Table 4 MAGE-1 expression in non-malignant ovarian lesions

| Histology                     | Number of specimens | MAGE-1 positive |
|-------------------------------|---------------------|-----------------|
| Normal                        | 16                  | 1               |
| Inclusion cysts               | 5                   | 3               |
| Serous cystadenoma            | 4                   | 2               |
| Mucinous cystadenoma          | 3                   | 1               |
| Pseudomyxoma                  | 1                   | 0               |
| Serous borderline             | 2                   | 0               |
| Mucinous borderline           | 1                   | 0               |
| Fibroma                       | 3                   | 3               |
| Endometriosis                 | 2                   | 2               |
| Dermoid                       | 3                   | 0               |

### Table 5 MAGE, BAGE and GAGE gene expression in malignant ovarian lesions as determined by RT-PCR

| Histology          | Number of specimens | MAGE-1 | MAGE-2 | MAGE-3 | MAGE-4 | BAGE | GAGE -1, -2 | GAGE -3, -6 |
|--------------------|---------------------|--------|--------|--------|--------|------|-------------|-------------|
| Serous             | 14                  | 10     | 0      | 0      | 1      | 0    | 1           | 0           |
| Mucinous           | 7                   | 2      | 1      | 0      | 0      | 0    | 1           | 1           |
| Endometrioid       | 2                   | 0      | 0      | 0      | 0      | 0    | 0           | 0           |
| Granulosa cell     | 2                   | 2      | 0      | 0      | 0      | 0    | 0           | 0           |
| Ovarian secondary  | 2                   | 1      | 0      | 0      | 0      | 0    | 0           | 0           |
| Metastases         | 6                   | 2      | 0      | 0      | 0      | 0    | 0           | 0           |
during the cyclical proliferation required for ovulation and repair, but not during the period of ovarian quiescence that occurs at the climacteric. This of course suggests that MAGE-1 gene expression does not play a role in carcinogenesis at all, rather in cellular proliferation.

Our findings in malignant ovarian tissue may also be highly relevant. Serous cystadenocarcinomas are the largest histological class of ovarian cancer and it is in this tumour category that we have shown preferential expression of MAGE-1. In addition, we report that the frequency of expression in this tumour type is greater in early-stage lesions. The finding of preferential expression in this tumour type is supported by other investigators (Yamada et al. 1995). However Yamada et al reported more frequent expression in later stage lesions. The discrepancy between these reports is probably a reflection of the small number of primary serous cystadenocarcinomas analysed in each series – a total of 14 lesions in this report and 13 in the series reported by Yamada. Another report shows higher frequency of BAGE and GAGE expression in ovarian tumours (Russo et al. 1996), although direct comparison with the results presented in this paper is not straightforward and the disparity is quite possibly due to methodological differences, e.g. increasing the number of PCR cycles could potentially increase the frequency of gene expression.

The finding of MAGE-1 gene expression in putative precursor lesions and early-stage serous cystadenocarcinomas could be interpreted as evidence that MAGE gene expression is an early as opposed to late event in ovarian tumour carcinogenesis. This study shows preferential MAGE-1 gene expression in ovarian serous cystadenocarcinomas. There is therefore potential to include this tumour type in future MAGE-1 vaccine trials. In addition we have shown MAGE-1 gene expression in a variety of non-malignant ovarian lesions – preferential expression occurring in those lesions obtained from premenopausal women. This finding questions previous assumptions regarding the role of the MAGE gene family in carcinogenesis and contributes to the growing body of knowledge concerning the natural history of ovarian carcinoma.

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