Cancer-testis antigen expression in bladder cancer

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INTRODUCTION

Superficial transitional cell carcinomas (TCC) account for about 70% of all bladder cancers in North America and Europe [1]. Following surgery, these tumours recur in more than 60% of cases and up to 25% of recurrences become invasive. For more than 20 years, intravesical bacillus Calmette-Guérin (BCG) instillation has been used successfully to prevent recurrence and progression of superficial TCC after surgery, or for the treatment of carcinoma in situ. Nearly 50% of patients at high risk of recurrence remain free of disease after receiving BCG immunotherapy following surgery. This suggests that immunotherapeutic treatments targeting specific tumour-associated antigens might be more effective and associated with less morbidity than BCG. Bladder cancer thus offers a unique opportunity to develop cancer vaccines in an ideal setting of minimal tumour burden. However, the development of specific immunotherapy depends on the identification of relevant target antigens. Cancer-testis antigens (CTAs) constitute one of the most interesting class of tumour-associated antigens [16,19]. They are immunogenic proteins expressed in various types of cancers. Their expression in normal tissues is restricted to germ cells in the testis and is occasional in non-gametogenic tissues. As testis tissues are immunoprivileged by their lack or low expression of HLA molecules, CTAs appear as ideal targets for immunotherapy. Moreover, the identification of CTA epitopes recognized by CD4+ and CD8+ T-cells and the high-titer antibody responses observed against some CTAs show their relevance in the immune response against cancer. However, a major drawback to their use as targets is their heterogeneous expression [10]. Successful CTA-based immunotherapy would thus likely be achieved by use of multiantigenic vaccines.

Expression of CTA genes appears to be mostly regulated by DNA methylation and their expression in neoplastic tissues seems to correlate with the frequent cancer-associated hypomethylation [19]. Previous studies tested new classes of chemotherapeutic agents inhibiting DNA methylation or histone deacetylation (HDAC) to induce CTAs expression in tumour cells. The methylation inhibitor 5-aza-2’-deoxycytidine (5-AZA-DC) [4], alone or in combination with a HDAC inhibitor such as Trichostatin A (TSA) [14], was shown to efficiently induce expression of several CTAs in cancer cell lines [5,8]. Thus, these drugs used in association with an appropriate vaccination strategy targeting CTAs could increase the expression of CTAs in patients, making them potentially useful for cancer immunotherapy.

RÉSUMÉ


Matériel et Méthodes: L’expression de NY-ESO-1/LAGE-1, MAGE-A, MAGE-C1, BAGE, HOM-TES-85, SCP-1, SSX-1, SSX-2 et SSX-4 a été analysée par RT-PCR semi-quantitatif dans 10 urothéliums normaux, 24 tumeurs superficielles, 22 tumeurs infiltrantes et dans 10 lignées vésicales traitées par 5-aza-2’-déoxycytidine (5-AZA-DC) et/ou Trichostatin A.

Résultats: L’expression de tous les gènes des CTA a été observée dans au moins une tumeur à l’exception de HOM-TES-85 qui n’a jamais été détecté. Les ARNm de MAGE-A, BAGE et NY-ESO-1/LAGE-1 ont été les plus fréquemment détectés, dans respectivement 67%, 21% et 8% des tumeurs superficielles et dans 64%, 41% et 27% des tumeurs infiltrantes. À l’exception de MAGE-A, les transcrits CTA ont rarement été détectés dans les lignées vésicales. Cependant, l’expression de tous les gènes des CTA sauf SCP-1, a été variablement induite par les drogues, 5-AZA-DC étant un inducteur beaucoup plus puissant que le TSA.

Conclusion: Ces données suggèrent que les CTA pourraient être utilisés comme cibles dans l’immunothérapie du cancer de la vessie, surtout ceux qui sont souvent exprimés comme MAGE-A, BAGE et NY-ESO-1/LAGE-1. De plus, l’induction de ces antigènes par des agents de chimiothérapie tel le 5-AZA-DC, offre la possibilité d’un prétraitement augmentant l’immunogénicité des tumeurs.

Mots clés : Cancer, vessie, antigènes testiculaires du cancer, MAGE, NY-ESO-1, immunothérapie.

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number of tumours eligible for immunotherapy and help to improve the immune response.

Previous studies on CTAs expression in bladder tumours focused on the MAGE-A or NY-ESO-1 families but none reported simultaneous analysis of many CTAs in one panel of tumours [2,11,12,15,17]. In the present study, we examined the expression of 9 CTA genes or families of genes in superficial and invasive TCCs. Moreover to establish a strategy for chemoimmunotherapy, we assessed the potential of 5-AZA-DC and TSA to induce the expression of CTAs in bladder cancer cell lines.

**MATERIAL AND METHODS**

**Clinical specimens**

This study was approved by the Institutional Review Board of CHUQ-l’Hôtel-Dieu de Québec. Bladder tumour specimens were collected between 1984 and 1990, frozen in liquid nitrogen and stored at −80°C. Normal urothelial mucosa were isolated from the bladder of organ donors. Normal testis specimens were also obtained from organ donors.

**Cell lines**

Mycoplasma-free bladder cancer cell lines (MGH-U3, SW780, RT4, 5637, 639V, T24, VMUCB-3, J82, JON and SW1710) were cultured in Minimal Essential Medium (MEM, Gibco/BRL, Burlington, ON) containing 10% foetal calf serum. 5-AZA-DC (Sigma Chemical Company, St-Louis, MO) and TSA (Sigma) treatments were carried out in T25 tissue culture flasks on 25-50% confluent cells, twenty-four hours after plating. Cells were treated for 48 hours with 1 µM 5-AZA-DC and/or 0.5 µM TSA. Alternatively, after 48 hours of 5-AZA-DC treatment, cells were treated for an additional 48 hours with 5-AZA-DC alone or with TSA.

**RNA and protein extractions**

RNA and proteins were isolated from frozen tumour specimens or cultured cells using TRIzol (Invitrogen, Burlington, ON). Proteins were quantified using the Bradford method following the manufacturer’s recommendations. RNA was quantified by optical density at 260 nm and its quality assessed on formaldehyde-agarose gels. The presence of normal or near normal proportions of 28S and 18S ribosomal RNAs attested to the quality of the specimens. DNA contamination was eliminated using DNase I (2 units/10 µg RNA, Ambion, Austin, TX). The enzyme was inactivated with the DNase Inactivating Reagent (Ambion).

**Reverse-transcriptase polymerase chain reaction (RT-PCR)**

RT reactions were performed at 37°C for 1 h using 1 µg of DNA-free RNA and 200 units of M-MLV reverse transcriptase in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 0.5 mM dNTP, 10 mM DTT, containing 100 ng of hexanucleotide primers and 25 units of RNA Guard (Amersham Biosciences, Baie d’Urfé, QC) in a final volume of 20 µl.

PCR primers for the human ß-ACTIN and CTA genes were selected using the Prime function of GCG (Wisconsin Package Version 10.2, Genetics Computer Group, Madison, Wisc.) (Table 1). Specificity of MAGE-A primers was confirmed using plasmids containing full-length cDNAs provided by Dr. De Plaen (Brussels, Belgium) (MAGE-A1, A2, A3, A4, A6, A12) or purchased from Invitrogen (MAGE-A8, A9, A10, A11). PCR amplification of ß-ACTIN transcripts was first performed to select the cDNA quantity limiting the amplification to the exponential phase. CTA gene expression was measured using 300 times more cDNA than for ß-ACTIN. PCR reactions were performed using up to 5 µl of cDNA and 0.5 units of Platinum Taq DNA polymerase (Invitrogen) in PCR buffer containing MgCl₂, 0.2 µM of both primers, 0.2 mM dNTP, 0.1 mM dATP and 2.5 mCi of dATP-[a32P] (3000 Ci/mmmole, Mandel Scientific Co. Ltd., Guelph, ON). PCR reac-

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**Table I: List of primers and amplification conditions used to analyze the expression of ß-ACTIN and 9 CTA genes or families of genes.**
tions were conducted in a Perkin Elmer 9600 thermocycler (Nor-
walk, CT) as follows: 95°C for 5 min followed by 28 (for β-
ACTIN) or 32 (for CTA genes) cycles of 95°C (15 sec) - 52-57°C 
(30 sec) - 72°C (30 sec) with final extension at 72°C for 10 min. 
MgCl2 concentrations and annealing temperatures are indicated in 
Table I. After drying, electrophoresis gels were exposed to a 
phosphor screen and analyzed with a PhosphorImager Storm 860 
(Molecular Dynamics, Sunnyvale, CA). Band intensity was evalu-
ated using the ImageQuant software (Molecular Dynamics) to 
determine CTA/β-ACTIN ratios. Ratios between 0.13 and 0.9 were 
considered to represent low expression, those between 0.9 and 2.5, 
medium expression and those ≥ 2.5, high expression. Ratios below 
0.13 were considered as background and not relevant.

Western blots
Thirty µg of tumour proteins or 15 µg of cultured cells proteins were 
separated on SDS-PAGE and transferred onto Hybond C nitrocellu-
lose membranes (Amersham BioSciences). After blocking, filters 
were incubated at room temperature for 1-2 h with monoclonal anti-
bodies (mAb) 57B to MAGE-A or D8.38 to NY-ESO-1 (dilution 
1:100), provided by Dr Spagnoli (Basel, Switzerland), or with a 
mouse polyclonal against human β-ACTIN (Sigma) [10]. Membranes 
were washed and incubated for 1 h with horseradish-labelled 
goat anti-mouse secondary antibody (Jackson Immunoresearch 
Laboratories, West Grove, PA). Bound antibodies were revealed 
using enhanced chemiluminescence (Perkin Elmer).

RESULTS
To maximize mRNA detection from clinical samples with various 
levels of integrity, random primers were used rather than oligo-dT 
for RT reactions and primers for 80-140 bp amplicons were selec-
ted. Primers for non-familial CTA genes such as SCP-1 and HOM-
TES-85, for the first member of the MAGE-C family, MAGE-C1

Figure 1: Expression of CTA genes in superficial (Ta-T1) and invasive (T2) bladder tumour samples. Semi-quantitative RT-PCR results are expressed as ratios between CTA and β-ACTIN gene expressions. White cases : No expression; Yellow cases : Low expression; Blue cases : Medium expression; Red cases : High expression. NY-ESO-1* (NY-ESO-1/LAG-1)
(CT7), for members of the SSX gene family, SSX-1, SSX-2, SSX-4, for BAGE-1, -4 and -5 but not for BAGE-2 and -3, for NY-ESO-1 and LAGE-1a but not LAGE-1b and finally for multiple members of the MAGE-A family i.e. MAGE-A1, A2, A3, A4, A6, A8, A9, A12 but not A10 and A11 were used as described in Table I.

**CTA expression in clinical specimens**

No significant expression of any CTA genes was detected in 10 normal urothelia except for MAGE-A which gave a very weak signal in 7 out of the 10 samples (results not shown). Exceptionally, the threshold of low reactivity for MAGE-A genes was increased from 0.13 to 0.30 to show the overexpression of these genes in the tumours compared to the normal tissues. Figure 1 shows the results of CTA gene expression analysis in the 24 superficial (6 Ta and 18 T1), 22 invasive bladder tumours (9 T2, 10 T3 and 3 T4) and one testis specimen. Expression of all CTA genes or families of genes was detected in at least one bladder tumour sample, with the exception of HOM-TES-85. Sixteen (35%) tumours did not express any of the CTA genes analyzed. Thirteen (28%) tumours expressed only 1 CTA gene while the remaining 17 (37%) expressed up to 6 CTA genes. The expression of CTA genes was generally 2-3 times more frequent in invasive than in superficial tumours.

**Figure 2: Western blot analysis of the expression of NY-ESO-1 and MAGE-A proteins.** Proteins were extracted from the same bladder tumour samples used for RT-PCR analyses presented in Figure 1, separated on SDS-PAGE, transferred to nitrocellulose membranes and tested with mAbs 57B (anti-MAGE-A) and D8.38 (anti-NY-ESO-1).
Figure 3: Expression of CTA genes in bladder cancer cell lines treated or not with methylase and/or histone desacetylase inhibitors. Semi-quantitative RT-PCR results are expressed as ratios between CTA and β-ACTIN gene expressions. Treatment conditions were: 1, Control (untreated cells); 2, Cells treated with 1µM 5-AZA-DC for 48 h; 3, Cells treated with 0.5 µM TSA for 48 h; 4, Cells treated with 1µM 5-AZA-DC and 0.5 µM TSA for 48 h; 5, Cells treated with 0.5 µM 5-AZA-DC for 96 h; 6, Cells treated with 1 µM 5-AZA-DC for 96 h and with 0.5 µM TSA for the last 48 h. White cases: No expression; Yellow cases: Low expression; Blue cases: Medium expression; Red cases: High expression. NY-ESO-1* (NY-ESO-1/LAGE-1). N.D.: Not determined.
except for MAGE-A genes, which were also the most frequently expressed, in 67% of superficial and 64% of invasive tumours. About 70% of MAGE-A-positive tumours showed a medium to high expression. MAGE-A expression was not associated with grade as 1/2 G1 (50%), 15/21 G2 (71%) and 14/24 G3 (58%) tumours were positive. The second CTA transcripts most frequently expressed were BAGE in 21% of superficial and 41% of invasive tumours. However, more than 50% of the positive tumours showed only a weak expression. Expression of BAGE correlated with grade as 0/2 G1, 4/20 G2 (15%) and 10/24 G3 (42%) (p=0.035, chi-square) tumours were positive. The third most often detected CTA were NY-ESO-1/LAGE-1, expressed at high or medium level in 8% of superficial and 27% of invasive tumours. Three tumours expressed NY-ESO-1/LAGE-1 mRNA more strongly than the testis. As for BAGE, NY-ESO-1/LAGE-1 expression increased with tumour grade as it was found in 0/2 G1, 2/20 G2 (10%) and 6/24 G3 (25%) G3 tumours. This association was however not statistically significant. The other CTA genes analyzed were less frequently expressed, in at most 9% of bladder tumours.

In order to correlate the expression of CTA mRNAs measured in tumour samples with that of proteins, we performed Western blot analyses on total proteins, isolated from these same tumours. These analyses could be performed only for MAGE-A and NY-ESO-1 antigens as antibodies for the other CTA were not available to us. Figure 2 shows Western blots obtained with mAbs 57B to multiple MAGE-A antigens and D8.38 to NY-ESO-1 antigen. NY-ESO-1 antigen expression detected with mAb D8.38 paralleled mRNA expression. However, only 16/30 (53%) MAGE-A mRNA-positive tumours were positive with mAb 57B.

**Cell lines**

Nine TCC and one adenocarcinoma cell lines were tested for expression of CTA genes. To assess a possible induction of expression of the various antigens, cells were treated with 5-AZA-DC (inhibitor of methylation) and/or with TSA (inhibitor of HDAC) for 48 or 96 hours, as described. For some cell lines, no data could be obtained after 96 hours because of drug toxicity or because RNA was lost.

Four cell lines expressed none of the CTA mRNAs whereas 5 others expressed only MAGE-A (Figure 3). In addition to MAGE-A, the JON cells also expressed BAGE and SSX-4 mRNAs. Treatment with 5-AZA-DC and/or TSA induced the expression of all CTA genes or families of genes with the exception of SCP-1. For each cell line, expression of at least 6 and up to 8 CTA genes was induced. In general, expression was more efficiently induced by 5-AZA-DC than by TSA. The strongest inductions were observed for MAGE-A, MAGE-C1, SSX-1 and SSX-2 genes. However, in most cases, the level of expression observed in testis could not be attained.

Western blotting with mAb D8.38 showed a weak induction of the NY-ESO-1 antigen only in the 639V, VMUCB-3 and SW1710 (Figure 4). A strong induction of the MAGE-A antigens, as detected by mAb 57B could be observed following treatment with 5-AZA-DC and TSA. It paralleled perfectly mRNA expression at the exception of the RT4 cell line which showed no reactivity with mAb 57B despite medium mRNA expression.

**DISCUSSION**

We evaluated the expression of 9 CTA genes or families of genes in bladder tumours and normal urothelia to evaluate their potential as targets for specific immunotherapy. MAGE-A genes were the most frequently expressed with overall 65% of both superficial and invasive tumours. Patard et al. analysed the expression of MAGE-A1, -A2, -A3, -A4, -A6, -A8, -A9 and -A12. Patard et al. analysed the expression of MAGE-A1, -A2, -A3 and -A4 genes in 29 superficial and 28 invasive bladder tumours, using RT-PCR [15]. They found that 61% of invasive tumours expressed at least one of these MAGE-A genes, a result comparable to our own 64%. However, they only found 28% of superficial tumours expressing any of these genes, in contrast to the 67% we detected. This difference suggests that about half of our
positive superficial tumours expressed MAGE-A genes other than MAGE-A1, -A2, -A3, -A4, in agreement with the reactivity of only 9/16 (56%) mRNA-positive tumours with mAb 57B. This mAb was shown to preferentially recognize MAGE-A4-positive tumours although it was shown to efficiently react with MAGE-A1, -A2, -A3, -A4, -A6, and -A12 antigens in specificity analyses using transfected cells [13]. A higher level of expression of MAGE-A4 compared to other MAGE-A antigens has been proposed to explain the preferential reactivity of 57B. More recently, Bar-Haim et al. reported the overexpression of MAGE-A8 mRNA in 70% of bladder tumours [2]. These results suggest that MAGE-A8 could be the MAGE-A mRNA observed in our 57B-negative tumours. However we obtained evidences that other specific MAGE-A transcripts are also expressed in these tumours (unpublished data). A comprehensive analysis of the expression of MAGE-A antigens in bladder tumours will require the use of specific primers for each MAGE-A family member.

The second CTAs most frequently expressed in our panel of tumours were BAGE-1, -4 and -5. Their mRNAs were detected in 21 % of superficial and 41% of invasive bladder tumours. These data agree with those of Boel et al. who found that 30% of invasive bladder carcinomas expressed BAGE [3]. However in more than 50% of positive tumours the level of expression was low compared to that observed in testis.

NY-ESO-1 is one of the most immunogenic known antigens. Nearly 50% of patients with NY-ESO-1 positive tumours have detectable humoral immune response and CD8+ T-cell reactivity against this antigen [9,18]. We found NY-ESO-1/LAGE-1 to be the third most frequently expressed CTAs, in 8% of superficial and 27% of invasive tumours. Kurashige et al. [12] reported NY-ESO-1 gene expression in 28% of superficial and 38% of invasive tumours while Shamma et al. [17] found NY-ESO-1 and/or LAGE-1 expression in 14% of low-grade and 48% of high-grade bladder tumours. The higher rates of expression observed by these investigators may be explained by their amplification parameters. In our study, we chose to avoid saturation of signals to limit amplification of weak, less significant signals. We feel that the perfect match we observed between our RT-PCR and Western blot data attested to the validity of this strategy. Other CTAs were expressed in less than 10% tumours with SCP-1 and HOM-TES-85 being the less frequently expressed with respectively only 1 or no positive tumour.

The results presented in this study suggest that NY-ESO-1/LAGE-1, BAGE but mostly MAGE-A antigens could be relevant targets for bladder cancer immunotherapy, especially for superficial tumours. Several clinical trials assessing the efficacy of immunotherapy targeting CTAs (mostly MAGE-A3) in patients with various types of cancers, including bladder cancer, have already been reported. Generally speaking, their success has been limited, with less than 20% of patients showing regression after vaccination [7]. The failure to obtain clinical responses may have two major causes: failure to induce a significant T-cell response or resistance of the tumour to immunological attack. While the first cause may be eventually resolved by more efficient vaccine formulations, the second one will need better characterization of the patients’ tumour and immune system status. One mechanism developed by tumours to escape immune attack is outgrowth of antigen-loss or antigen-negative variants. Heterogeneous expression of CTAs in tumours is a condition favouring tumour escape [10]. In this study we evaluated the potential of 5-AZA-DC alone or in combination with TSA to induce the expression of CTA genes in bladder cancer cell lines. We showed that all CTAs analysed except SCP-1, could be induced by 5-AZA-DC. However, this induction raises questions about the ability of tumour cells to properly present induced tumour epitopes by HLA class I. Coral et al. [5] showed that de novo expression of NY-ESO-1 in 5-AZA-treated renal carcinoma cells could lead to efficient lysis by NY-ESO-1-specific cytotoxic T lymphocytes and that such treatment may also lead to increased expression of HLA class I and co-stimulatory molecules [6]. Thus, the use of 5-AZA-DC, also known as Decitabine, with an immunisation strategy aimed at inducing an efficient immune response against CTA antigens could provide an opportunity to 1) increase the number of tumours potentially treated by chemoimmunotherapy, 2) improve antigen presentation by HLA class I antigens and 3) possibly counter tumour escape mechanisms by inducing antigen expression in antigen-loss or antigen-negative tumour cell variants.

CONCLUSIONS

The BAGE, the NY-ESO-1/LAGE1 but mostly the MAGE-A families of antigens are the most frequently CTA expressed in bladder tumours and thus constitute targets of choice for bladder cancer immunotherapy. A more detailed analysis is needed to identify which specific MAGE-A antigens are expressed in bladder tumours. We showed that in bladder cancer cell lines the expression of these CTA can be induced by 5-AZA-DC. Thus a treatment combining immunotherapy and 5-AZA-DC chemotherapy could increase the number of tumours eligible for immunotherapy and help to improve the immune response.

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