Abstract. Malignant pleural mesothelioma (MPM) is a highly malignant tumor arising in patients previously exposed to asbestos fibers. Its increasing incidence and its social, financial and human impact have become a frequent problem in many industrialized countries. The unresponsiveness of malignant mesothelioma to conventional therapies has led clinicians to develop new treatments. As immunotherapy has been shown to offer promising and targeted treatment of MPM patients, the knowledge of the immunoresistance level of MPM may be a valuable tool for "à la carte" therapy. In a previous work, we profiled the gene expression of two MPM tissues compared to healthy mesothelial cells using a 10K cDNA microarray. Subsequent clustering analysis identified several clusters of differentially-expressed genes among those that are functionally-related to the immune system. In this report, we focus on genes with expression changes that may facilitate tumor escape from immune-mediated rejection. We also analyzed the immune reaction by staining the immunocompetent cells surrounding the tumor. Interestingly, the tumor with the strongest escape response, as shown by the expression of numerous immunoresistance-associated genes, displayed the strongest T cell infiltrate. The main genes conferring immunoresistance are CD74, HLADOA, HLADMB, PTGS1, IGFBP7 and TGFB3, by favoring immune tolerance, and CFLAR, DFFA, TNFRSF6, BNIP3L, by impairing apoptosis. These observations have fundamental consequences in the understanding of immunological properties of MPM, and offer a new insight into the mechanisms whereby MPM may circumvent host-mediated immune activities and promotes its own development. For an immunomodulation strategy to cure mesothelioma, it is crucial to characterize the MPM "immune signature" to design adapted immunotherapies.

Malignant mesothelioma is a solid serosal tumor with an etiology that has been strongly linked to previous asbestos exposure (1). The most common form starts in the chest cavity and is called malignant pleural mesothelioma (MPM). The clinical management of patients with MPM is difficult (2). In humans, malignant mesothelioma is an aggressive cancer characterized by a high local invasiveness, poor prognosis and therapy outcomes. By the time that malignant mesothelioma is diagnosed, the disease is often at an advanced stage, with a mean survival time of 12 months and less than 5% survival at 5 years (3). None of the conventional approaches, including surgery, chemotherapy and radiotherapy, has proven efficient in the treatment of malignant mesothelioma (4). This may partly be explained by lack of knowledge regarding the pathogenesis of malignant mesothelioma. Methods that allow simultaneous analysis of multiple molecular events, especially microarray technologies, have been applied to elucidate the complex network of cellular regulatory pathways altered in pleural carcinogenesis and how they participate in the generation of the tumor phenotype (5, 6).

In our laboratory, cDNA expression was recently used to compare the transcriptome of a MPM cell line (7) and two MPM tissue homogenates (8) to non-malignant mesothelial cells, leading to the characterization of several hundreds of
Table I. Features of the used primary antibodies.

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Tissue block embedding</th>
<th>Clone</th>
<th>Source</th>
<th>Titer</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 (T cell)</td>
<td>paraffin</td>
<td>NCL CD3p</td>
<td>Novocastra, Tebu-bio S.A.</td>
<td>1:200</td>
<td>20 min</td>
</tr>
<tr>
<td>CD4 (T helper)</td>
<td>paraffin</td>
<td>OCT0 M0756</td>
<td>Dako S.A.</td>
<td>1:100</td>
<td>20 min</td>
</tr>
<tr>
<td>CD8 (T cytotoxic)</td>
<td>paraffin</td>
<td>OCT M7103</td>
<td>Dako S.A.</td>
<td>1:50</td>
<td>20 min</td>
</tr>
<tr>
<td>CD20 (B cell)</td>
<td>paraffin</td>
<td>OCT M0755</td>
<td>Dako S.A.</td>
<td>1:200</td>
<td>20 min</td>
</tr>
<tr>
<td>CD22 (B cell)</td>
<td>OCT</td>
<td>M738</td>
<td>Dako S.A.</td>
<td>1:20</td>
<td>20 min</td>
</tr>
</tbody>
</table>

Immunohistochemistry was performed according to the manufacturers’ instructions from: ìOptimum Cutting Temperatureî medium; ìLe Perray en Yvelines, France; îTrappes, France; îLe Pont de Claix, France.

differentially-expressed (DE) genes. Moreover, the DE genes were clustered in (i) enhanced tumor invasiveness, (ii) macromolecule protection, and (iii) resistance to anticancer defenses. In spite of numerous similarities (shared DE genes), each MPM specimen displays its own gene expression profile (unique DE genes), thus offering a unique molecular fingerprint. Among genes that significantly discriminated MPM tissue transcriptomes, we marked those that are functionally related to immunity. In this study, MPM immunobiology was examined by re-analyzing clusters of immune-related genes. Moreover, the data presented here showed that variations in the "immune signature", found by comparing both MPM transcriptomes, actually reflect the presence of infiltrating immunocompetent cells, especially lymphocytes as assessed by immunohistochemical analysis.

Various experimental as well as clinical studies have evaluated immunomodulatory therapies and suggest their promising potency in the fight against mesothelioma (9). However, such therapy has to take into account the intrinsic immunoresistance of tumors. Interestingly, our analysis of immune-associated clusters of DE genes focuses on cellular and molecular mechanisms whereby mesothelioma may escape the host immune system control.

Materials and Methods

Samples and RNA isolation. Two specimens of human MPM, respectively "Fa" and "Sp", were obtained from CHU Cote de Nacre (Caen, France). Both matched the epithelial histotype of MPM (Galateau-Salle F., personal communication) and were snap-frozen in liquid nitrogen until use. The mesothelioma MSTO-211H (#CRL-2081) and the mesothelial MET-5A (#CRL-9444) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown to confluence, as recommended by the manufacturer. Total RNA was extracted from both ground tissues and the cells harvested using the Trizol™ Reagent procedure (GibcoBRL, Grand Island, NY, USA). Oligotex™ (Qiagen S.A., Courtabeuf, France) purified mRNA (1 µg each) was fluorescently-labelled by reverse transcription with either Cy3-dCTP or Cy5-dCTP and compared on microarray.

Microarray gene expression profiling. We profiled the gene expression in MPM tissues and cell lines using the Human UnigemV™ cDNA microarray (IncyteGenomics, Palo Alto, CA, USA), that allowed the screening of 9,984 human genes. In our experimental design, the mesothelioma cell line MSTO-211H (Cy3-labelled mRNA) served as the common standard for indirect comparison of MPM tissues with mesothelial cells MET-5A (both Cy5-labelled mRNA), as described previously (8). Each tissue sample was analyzed by four independent experiments, including tissue grinding, total RNA extraction, mRNA isolation and microarray hybridization.

The microarray results were analyzed by hierarchical clustering methods, either supervised with Gemtools™ software (IncyteGenomics) or unsupervised with Eisen’s Cluster/Treeview software (http://rana.lbl.gov/EisenSoftware.htm). Before computing, we applied two filters to meet the validation criteria we had defined (8). Only genes with a Cy-fluorescent signal higher than 2.5-fold the background level and covering the spot area >40% were used to calculate the Cy5/Cy3 ratio. As the reproducibility of the data was ensured by performing four independent experiments with both "Fa" and "Sp" MPM, the second filter excluded all genes that did not display a validated fluorescent ratio in at least three experiments. We set a variation threshold of 2-fold (up or down) in at least three experiments to mark genes with significant difference in their gene expression levels. Validated genes were named and italicized according to their corresponding HUGO symbol (http://www.gene.ucl.ac.uk/riomenclature/) and may be downloaded from the website: http://www.imrs.fr/htm/le_tour_du_mesotheliome_en_6_969_genes.html

Immunohistochemistry. For immunohistochemical evaluation of tumor infiltrating lymphocytes, tissue samples from "Fa" and "Sp" MPM were embedded in paraffin and Tissue-Tek® Optimum Cutting Temperature™ (OCT) medium, respectively. Serial sections were cut and collected onto microscope slides. Before immunostaining, MPM "Fa" sections (paraffin-embedded) were deparaffinized through xylene and pretreated by autoclave incubation (2.5 min) with citrate buffer (pH 6.0). The MPM "Sp" sections (OCT-embedded) were further fixed by formalin-methanol-acetone treatment: formalin (pH 7.6) for 10 min at room temperature, methanol for 4 min at ~20°C, acetone for 2 min at ~20°C and acetone for 2 min at room temperature.

The presence of CD3 (pan-T lymphocytes), CD4 (T helper cells), CD8 (T cytotoxic cells) and CD20 or CD22 (B cells) antigens was tested in mesothelioma tissues based on the indirect immunoperoxidase staining procedure, using the avidin-biotin method from the Basic DAB Detection Kit (Ventana Medical Systems, Illkirch, France) in the Ventana ES™ automated immunostainer, following the manufacturer’s instructions. The characteristics of the primary anti-CD antibodies used are
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Figure 1. Gene cluster relevant to mesothelioma "Fa" immune signature. A cluster of MPM "Fa" overexpressed genes was extracted from the unsupervised hierarchical clustering of the MPM gene expression profile according to Eisen's Cluster/Treeview software. This is a part of the original gene dendrogram that contained 757 differentially-expressed genes. The differential expression of each transcript (row) in each experiment that contained 757 differentially-expressed genes (8). MPM "Fa" (4 experiments), MPM "Sp" (4 experiments) and mesothelial cells (MET-5A) samples were subjected to hierarchical clustering of the MPM gene expression profile according to Eisen's Cluster/Treeview software. This is a part of the original gene dendrogram that contained 757 differentially-expressed genes (8). MPM "Fa" (4 experiments), MPM "Sp" (4 experiments) and mesothelial cells (MET-5A) samples were subjected to hierarchical clustering of the MPM gene expression profile according to Eisen's Cluster/Treeview software. This is a part of the original gene dendrogram.
summarized in Table I. Sections from both MPM specimens were immunostained for each antibody in a single experiment. Briefly, the sections were first incubated with the primary specific antibody and next with a secondary biotinylated antibody. Antibody detection was performed using horseradish peroxidase conjugated to avidin in the presence of diaminobenzidine (DAB)/H₂O₂, yielding a red-brown precipitate of DAB/copper sulfate. The slides were counterstained with Harris' hematoxylin before mounting and evaluation of lymphocyte infiltration.

Results

Mesothelioma differentially-expressed genes. As detailed previously, we generated a 10K-gene expression profile from two MPM tissues by performing four independent cDNA microarray experiments (8). Sample replicates showed reliable reproducibility and validity for a subset of 5,402 genes, among which 757 were found differentially-expressed (DE) in MPM samples as compared to MET-5A mesothelial cells.

Hierarchical clustering analysis. Focusing on 757 DE transcripts, hierarchical clustering classified genes and samples according to their degree of gene expression similarity. Our analytical approach was validated since both analyzed MPM, “Fa” and “Sp”, clustered separately although four independent experiments were performed. Nevertheless, both MPM tissues showed sufficient similarities to form clusters clearly separated from MET-5A mesothelial cells (Figure 1 and ref. 8).

Hierarchical clustering of 757 DE genes also yielded robust clusters of DE genes that were relevant to characterize the MPM phenotype. Interestingly, both supervised and unsupervised clustering methods revealed clusters of expression changes of genes that functionally belong to tumor invasion processes, cellular protection and resistance pathways against anti-tumor defenses, i.e. patient immune system (endogenous defenses) and treatments (exogenous xenobiotics). Many of these clusters, except for the one related to immunity, that are analyzed in this paper, were extensively analyzed in our previous paper (8).

Immune-associated cluster of DE genes. We identified two sets of overexpressed genes consistent with immune functions. The first one, outlined in (8), contained mainly genes encoding adhesion and recognition proteins involved in both the invasion and immune processes. As exemplified in Table II, immune-related genes included many HLA class II-encoding genes that function in the molecular processing for antigen presentation and several genes encoding immune cell receptor, namely FCGR3A, FCGR3B, LILRB5 and SDF1R (underlined in Table II). A literature search showed that several of those genes, outlined in Tables II and III, may facilitate tumor progression by acquiring defenses against immunity, as discussed below.

<table>
<thead>
<tr>
<th>Immunity function</th>
<th>DE</th>
<th>HUGO approved names of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T cells</strong></td>
<td>Over</td>
<td>CD48* (fa); CD64* (fa); GZMA* (fa); GZMK (fa); MCP2* (fa); MAL* (fa); SYK* (fa); TRB* (fa); GZMK (sp); ILF3 (fa);</td>
</tr>
<tr>
<td></td>
<td>Under</td>
<td></td>
</tr>
<tr>
<td><strong>Complement pathway</strong></td>
<td>Over</td>
<td>BF (co); C1QBP (co); CIQR1 (fa); C1S (co); C2 (fa); C5 (sp); C4A3R1 (fa); CFH (fa);</td>
</tr>
<tr>
<td></td>
<td>Under</td>
<td></td>
</tr>
<tr>
<td><strong>Monocytes and macrophages</strong></td>
<td>Over</td>
<td>CD14* (fa); CD163* (fa); MARCO* (fa); MNDA* (fa); STAT1* (fa); TNFSF13* (fa);</td>
</tr>
<tr>
<td></td>
<td>Under</td>
<td>LAGL3 (co);</td>
</tr>
<tr>
<td><strong>Adhesion and recognition</strong></td>
<td>Over</td>
<td>FCGR1A* (fa); FCGR3A (co); FCGR3B (co); ICAM1 (fa); ITGAM* (fa); ITGAX* (fa); LAIR1* (fa); LILRA3* (fa); LILRB4* (fa); LILRB5 (fa); SELL* (fa);</td>
</tr>
<tr>
<td></td>
<td>Under</td>
<td></td>
</tr>
<tr>
<td><strong>Cytokines and receptors</strong></td>
<td>Over</td>
<td>SCYA11 (fa); SCYC1* (fa); SDF1 (co); SDF1 (fa);</td>
</tr>
<tr>
<td></td>
<td>Under</td>
<td>IL1A (fa);</td>
</tr>
<tr>
<td><strong>Antigen presentation</strong></td>
<td>Over</td>
<td>CD74 (fa); HLADMB (co); HLADNA (fa); HLADOA (fa); HLADOB (sp); HLADPA1 (co); HLADPB1 (co); HLADB1 (co); HLADRA (co); HLADRB1 (co); HLADRB3 (co);</td>
</tr>
</tbody>
</table>

Differential expression of genes: ‘over’, ‘under’ refer to overexpression or underexpression of both MPM (co), MPM (fa) or MPM (sp) as compared to mesothelial cells. Underlined genes are immune receptors. Asterisks (*) mark genes that belong to the immune-related cluster of genes specifically overexpressed in MPM “Fa” that are also displayed in Figure 1.
As shown on the color map, both tumors behaved differentially according to the second cluster (Figure 1). Indeed, MPM "Fa" significantly overexpressed most of these genes as compared to MPM "Sp". These genes (underlined in Figure 1) concerned mainly (i) immune effectors like T cells: CD8A, CD48, GZMA, LCP2, SYK and TRB@, (ii) the monocyte/macrophage lineage: CD14, CD 163, MARCO, MNDA, STAT1 and TNFSF13, as well as (iii) other immune-specific cell receptors and adhesion molecules: CYBB, FCGR1A, LILRB3, ITGAM, ITGAX, LAIR1, LILRA3, LILRB4 and SELL.

**T and B cells staining.** Immunohistochemical staining of cell surface markers was performed on MPM tissues to check the presence of infiltrating lymphocytes at the tumor site. As evidenced in Figure 2, sections of MPM "Fa" and MPM "Sp" stained strongly when an antibody against the T cell marker CD3 (panels A and B) was used, whereas little B cell staining was observed (panels C and D). Nonetheless, MPM "Fa" displayed a more intense and diffuse CD3 immunoreactivity compared to MPM "Sp" (Figure 2, panel A versus B), suggesting more T cells infiltrating the tumor mass. In addition, there was a starkly contrasting pattern of T cell infiltration between the two MPM tissues. CD8+ cells predominated in MPM "Fa" (Figure 2, panel E vs F), whereas CD4+ cells predominated in MPM "Sp" (Figure 2, panel H vs G). Taken together, these observations reflected tumor heterogeneity and suggested a more developed immune response for "Fa" MPM, although both tumors were typed as epithelial MPMs.

**Discussion**

Tumor immunology is dictated by the balance of immunological surveillance that specifies the host immune reactions against tumor cells, and immune escape, which refers to the tumor-cell evasion process against the host immune system. Indeed malignant mesothelioma immunobiology is currently under investigation as MPM has a poor prognosis whatever the treatment (10, 11). Malignant mesothelioma cell lines have been considered as weakly immunogenic but, in some circumstances, an immune response can take place. Robinson et al. (12) reported a case of transient spontaneous regression of MPM that correlated with mononuclear cell infiltration and serologic anti-mesothelioma reactivity. In that study, the tumor grew again following the loss of the serologic response. As a consequence, there is some hope that it will eventually be possible to modulate the immune system in order to induce an efficient anti-mesothelioma response. The current challenge in tumor immunology is to understand the critical balance between immune activation and immune inhibition with regard to the host-tumor interaction. Here, we discuss the relationships of immunohistochemical staining and microarray profiling and focus on some interesting results about defects in the development of an immune-mediated anti-tumor response.

**Immunological surveillance.** Regarding their gene expression profile, MPM tissues showed robust clusters of DE genes that could be matched with immune functions (Figure 1 and Table II). Those genes may be involved in the "immune signature", as their products may reflect some traits of the immune response to tumor cells. The "immune signature" of MPM appears relevant as our data showed differences in both analyzed profiles. Indeed the cluster presented in Figure 1 contains exclusively DE genes from MPM "Fa", that are involved in immunity. We supposed that such discrepancies in gene expression reflected changes at the cellular level. We, therefore, screened both MPM to check the distribution of immune T and B cells. As expected, the distribution of the CD3+ and CD8+ lymphocyte subpopulation was higher in MPM "Fa" than MPM "Sp" (Figure 2). Actually lymphocytic infiltration of the tumor tissue contributed to the gene expression profile of the whole tumor, as was shown in breast tumors (13).

Overall examination of the immune-related genes showed that genes overexpressed in both tumors function mostly in immune recognition (HLA system) and immunoresistance. In contrast, those overexpressed in only "Fa" MPM concerned a variety of immune effectors and functions, including T cell, macrophage, NK cell and complement components. Immune-related genes differentially-expressed only in MPM "Fa" may be related to tumor-infiltrating cells, whereas those commonly modulated in both MPM may specify the immune phenotype of mesothelioma cells.

**Immune escape.** Although tumors frequently express antigens recognized by the host immune system, they progress by evolving immunoresistant variants (14). According to Deichman (15), as well as to data we published previously (8), immunoresistance properties may contribute to the protective phenotype of MPM. Immune deficiency in cancer patients is well documented and is known to affect all components of the immune system (16). The data we obtained here suggest several scenarios that may decrease the efficiency of the immune response to MPM. They involved the induction of immune tolerance and the modulation of apoptosis pathways.

**Immune tolerance:** Several DE genes in this study (Table III) may either alter antigen presentation, affect the expression of the appropriate target antigen or self-antigen, produce immunosuppressor agents, or block antibody activity.

**Impaired antigen presentation:** Malignant mesothelioma cell cultures were shown to express constitutively high levels of HLA class I molecules, whereas the expression of class II
Figure 2. B and T cells infiltrate in mesotheliomas "Fa" and "Sp". Sections of MPM "Fa" (panels A, C, E, G) and MPM "Sp" (panels B, D, F, H) were immunostained with anti-CD3 (A and B), anti-CD20 (C) or anti-CD22 (D), anti-CD8 (E and F) and anti-CD4 (G and H) antibodies (magnification x40).
molecules was shown to require gamma-interferon stimulation (17). Some authors have detected constitutive MHC class II-DR on the primary mesothelioma cell surface (18). Our results indicated that (i) both mesothelial and mesothelioma samples highly-expressed the HLAB gene belonging to class I (8), and (ii) both MPM overexpressed a large panel of HLA class II-encoding genes (Table II). This profile is suggestive of a tumor microenvironment enriched in factors that stimulate class II expression. Some, if not all, gamma-interferon pathways may also be activated in MPM. Thus, in contrast to other tumors, the loss of HLA molecules may not explain the poor immunogenicity elicited by both analyzed MPM. However, several hypotheses could account for the lack of immunogenicity of tumoral cells, despite HLA class II molecule expression (19).

First, the invariant chain (ii) encoded by the CD74 gene (Table II), and needed for HLA class II antigen processing, can reduce the immunogenicity of HLA class II-expressing cells by impeding the loading of tumor peptides. Since the expression of the CD74 gene was increased in both MPM, our results are consistent with (i) an antigen presentation failure due to overexpression of both invariant chain and HLA class II genes, and with (ii) blockage of surface HLA class II dimers by the invariant chain-derived class II-associated Ii peptide (CLIP) (20).

Secondly, an abnormal antigen presentation could also result from alterations in the expression of non-classic HLA class II molecules, namely HLA-DO and HLA-DM, which control the peptide loading on HLA class II dimers. HLA-DM catalyzes peptide exchange between CLIP and the antigen on HLA class II molecules, while HLA-DO inhibits this activity. Both MPM overexpressed the HLADMB gene, whereas HLADOA and HLADNA were overexpressed in MPM "Fa" and HLADOB in MPM "Sp", respectively (Tables II and III). All belong to the HLA-DO gene family. Modulation of HLA-DM-dependent peptide loading may influence the peptide repertoire displayed to T cells by selective inhibition of CLIP removal or by restriction of class Il-binding to a specific, weakly immunogenic peptide.

Finally, HLA class II expression in the absence of the other accessory molecules, which are required in the "three-step event" process of activation by an antigen-presenting cell, leads to immune-cell anergy. Lack of co-stimulatory molecules of the B7 family was shown in established malignant mesothelioma cell lines (18). Inversely, B7.1 expression from a B7.1 expression vector enhances the immunogenicity of malignant mesothelioma cells, as shown by experiments carried out using a murine model (21). Thus, when HLA class II expression is inducible or constitutive in a solid tumor, the immune response could be impaired by either a defect of antigen processing or lack of co-stimulation.

**Lack of adapted tumor antigen:** Initially, tumor-associated antigens were isolated from melanomas because of their immunosensitivity. Then, several melanoma-associated antigens were found in other tumor types and turned out to have an almost exclusively tumor-specific expression pattern. Diminished expression of those antigens was correlated, in some studies, with disease progression (22). Interestingly, MPM "Fa" down-regulated two genes of the cancer/testis antigen superfamily, namely MAAT1 and PRAME, and both MPM down-regulated SSFA2 (Table III). Loss of several tumor-associated antigen accounts for the failure of the immune system to clear those tumors.

There are very few data about target antigen in the immune response of MPM in the literature, although some interesting results came from studies dedicated to the serological analysis of mesothelioma patients in order to find mesothelioma-directed antibodies. Robinson's group characterized many features of malignant mesothelioma
immunogenicity. The following conclusions are noted here: (i) almost 30% of mesothelioma patients exhibit immune reactivity and clearly react to the presence of the tumor (23), (ii) patients with a spontaneous mesothelioma regression showed dynamic changes in their serological response to tumor antigen, with a critical failure when disease progressed again (12) and, (iii) serological reactivity of mesothelioma patients allowed the identification of eight mesothelioma-associated antigens, some of which were normal proteins known to be overexpressed in growing tumors, including TOPIIB (24). In accord with the latest assertion, Robinson et al. hypothesized that a tumor may overexpress the gene encoding the product to which the patients made an antibody response. Our data confirmed this fact, since TOPIIB was found increased in both MPM (Table III). Tumor ‘Fa’ also expressed both MSLN and TPD52L1, respectively encoding mesothelin and the D52-like tumor-specific protein. Mesothelin is targeted by mesothelioma-specific K1 antibody, but is also present on mesothelial cells, epithelial ovarian cancer and on squamous cell carcinoma (25). Experimental investigations and the data mentioned above support the notion that the immunogenicity of a particular tumor is a consequence of overexpression of normal or oncofetal proteins, rather than the neo-expression of tumor antigens (23, 24). As a consequence, a defective immune response could be directly due to a lack of autoimmunity, if such self-components were expressed during T cell development in the thymus. Alternatively, when an autoimmune response does occur, the humoral response dominates and the anti-MPM antibodies activity is ineffective because MPM developed several antibody-related counterattacks, as detailed below.

**Immunosuppressive activities:** The maturation and activity of immune cells are also dependent on the mediator repertoire present in their vicinity. Malignant mesothelioma cells have been shown to secrete a variety of growth factors and cytokines, some of which drive the immune response towards tolerance or immunity (26). In our study, MPM produced an excess of several transcripts related to immunosuppression. As seen in Table III, they include genes involved in prostaglandin synthesis: PTGS1 (co), IGFBP7 (co), PLA2G2A (fa) and PTGIS (sp). Prostaglandins are expressed in many human tumors as a result of enhanced expression of the COX enzyme (PTGS genes), which is a rate-limiting enzyme for prostaglandin synthesis. It has been shown that PTGS2 expression has a prognostic value in malignant mesothelioma, a high level correlating with poor survival (27). The prostaglandin pathway has been shown to be involved in the growth of solid tumor by promoting cell survival, proliferation and invasion, but it also acts as an immunomodulator (28). For example, prostaglandin E2 acts together on T-, B- and antigen-presenting cells to favor the Th2 response impairing tumor destruction (28). This feature may explain why several patients display serological reactivity against MPM. Moreover, MPM ‘Fa’ up-regulated the IL4R and IL10RA genes (Figure 1 and Table III) encoding the receptors of, respectively, IL-4 and IL-10, both type-2 cytokines involved in the Th2 response that promotes either humoral immunity against tumor or immune deviation to tolerance (29). Novel signaling pathways for prostaglandin derivatives have been discovered, especially for prostaglandin I2 that displays a suppressive activity in inflammation, as well as proliferation and metastasis.

MPM ‘Sp’ overexpressed the TGFB3 gene (Table III). Transforming growth factor β proteins are produced at high level in malignant mesothelioma tumors or cell lines (30). Once in the tumor microenvironment, TGFB3 has been shown to be a major anergic factor by depressing the T cell immune response (31).

Despite cytokines, other molecules can have a suppressive effect on the development of immune response by blocking the antibody-dependent cell cytotoxicity. Both MPM overexpressed the FCGR3B gene encoding a receptor for the Fc region of gamma-immunoglobulin. In contrast to other immunoglobulin receptors, FCGR3B is not able to mediate antibody-dependent cytotoxicity and phagocytosis, but conserves its binding capacity against monomeric, complexed and aggregated gamma-immunoglobulin. It may act as a trapping molecule that prevents an effective antibody-dependent immune response by sequestering antibody produced against tumor antigens.

The complement system of innate immunity also has a potential role in fighting tumors through a predominant Th2 response and antibodies directed against MPM proteins. Several complement-related genes were found overexpressed in experiments we carried out (Table II and gene cluster in Figure 1). It has also been shown that tumors may develop protective mechanisms to hamper complement efficiency (32). Among the membrane-bound regulator of the complement, our previous study showed up-regulation of the CD59 gene in the mesothelioma cell line MSTO-211H (7) and, recently, we confirmed this result in MPM ‘Sp’ malignant microdissected cells (33). The CD59 protein interferes with the formation of the membrane attack complex. In the present study, MPM ‘Fa’ overexpressed two genes of soluble regulators of the complement: C1QB and CFH (Figure 1 and Table II). C1QB binds to the globular head of C1Q, the first component of the classical pathway, and inhibits complement-mediated lysis (34). The second gene encodes the complement factor H, a central regulator of the complement system, which participates in the inactivation of C3b and accelerates the dissociation of both C3- and C5-convertases in the alternative complement pathway. Indeed, complement factor H was related to resistance to complement in many tumor types (32).
Moreover, it was previously purified from MPM effusions, where it is thought to display chemotactic activity for monocytes (35). However, its precise involvement in mesothelioma biology remains to be elucidated.

Modulation of apoptosis pathways. Despite immune tolerance, dysfunction in apoptosis is regarded as a key event in the development of immuno resistance. In mammalian cells, apoptosis is orchestrated by the caspase network through either death receptor-dependent or mitochondria-dependent activation. Malignant cells have developed several mechanisms that prevent them from entering the programmed cell death pathway. This allows initiated cells to grow and increase their resistance to pro-apoptotic signals induced by chemotherapy, X-rays and immune effectors.

Defective death receptor signaling: The death receptor pathway is initiated by the TNFα family of cytokines, including TNFα, FASL and TRAIL, known to induce apoptosis of transformed cells. Members of the TNF family are also involved in the regulation of immune homeostasis and cell-mediated cytotoxicity (36).

The FAS/FASL pathway particularly emerged from expression data we analyzed. Tumor cells are currently resistant to FAS-mediated apoptosis. Indeed pleural samples, whether there are malignant or not, express constitutively and abundantly CFLAR and DFFA genes (Table III and ref. 8), both encoding key regulators of this death pathway. Fixing FASL causes rapid death-inducing signals by linking to FADD and procaspase-8 that connects the FAS receptor to the caspase cascade. CFLAR (also FLIP or c-FLICE) resembles procaspase-8, but without the active proteinase site. Therefore, although it can be recruited to the signaling complex, it cannot convey a death signal. Medema et al. (37) found that CFLAR expression in a tumor makes tumor cells resistant to apoptosis induced by death receptor activation and participates in immunoresistance to T cells. DFFA (also called DFF45) acts downstream in one of the ultimate events of apoptosis. Indeed DNA fragmentation is caused by the action of caspase-3 on the DFF40/DFF45 complex, by cleavage of the DFF45 inhibitor and by subsequent cleavage of the chromatin by the nuclease DFF40. In living cells, DFFA is one of the main inhibitors of apoptosis-dependent DNAse activity (38). In fact, the high expression level of DFFA in mesothelial cells could prevent apoptosis-resulting events by sequestering and inactivating the DFF40 nuclease. As many immunocompetent cells and anti-cancer drugs have been shown to enhance FAS/FASL expression on tumor cells, high expression of both intracellular inhibitors may counterbalance those cytotoxic effects. Indeed, FAS-induced apoptosis was inhibited in four of six MPM cell lines in a recent report (39).

It is noteworthy that the FAS-encoding gene, TNFRSF6, is overexpressed in MPM "Fa". As mentioned above, pleural cells may already be refractory to FAS-mediated cytotoxicity. Thus, the increased expression of TNFRSF6 in MPM "Fa" could be related to tumor infiltrating leukocytes. Beside initiating a T cell-induced apoptosis, FAS/FASL is particularly important in lymphocyte homeostasis by mediating activation-induced T cell death and infiltrated-cell death at immune privileged sites like testis or retina (40). The clearance of immune cells is dependent on FAS expression onto infiltrating cells and FASL onto the surrounding cells. Some tissues, that normally do not constitutively express FASL, up-regulate it only during potent immune response in the presence of activated T lymphocytes. Thus, like many tumors expressing FASL on their membrane, MPM may use this leukocyte death program to mount a FAS "counterattack" against tumor infiltrating immune cells, thereby inducing an "immune privilege" (41).

The TNF-related, apoptosis-inducer ligand or TRAIL kills transformed cells with specificity in vitro. Its inactivation may be one of the possible escape mechanisms against TRAIL-mediated apoptosis and may contribute to tumor development and progression, as was already reported in gastric cancer (42). Reduced expression of the TNFRSF10B gene (TRAIL-R2 or DR5) found in MPM "Sp" may also play a similar role in the pathogenesis of MPM. Moreover, the main cytotoxic effects of various immune cells are, at least partly, dependent on TRAIL expression (43). TRAIL-R2 is considered to be a p53 target gene that signals apoptotic death. Loss of this receptor, together with enhanced p53-related repair functions as mentioned above, may lead to tumor progression and resistance.

TNFAIP3, another TNF-related gene overexpressed in MPM "Fa", has been characterized as an inhibitor of both NF-ÎB and apoptosis. It belongs to the TNF primary response genes that have been shown to inhibit TNF-induced apoptosis, thus functioning as a negative feedback regulator. Instead, overexpression of TNFAIP3, together with those of SOD2 and PAI2, which both displayed enhanced expression in MPM "Fa" (8), may be an important factor of TNF resistance (44).

Disruption in mitochondria-mediated apoptosis: Mitochondria-dependent apoptosis is regulated by the balance of anti-apoptotic members of the BCL2 family and various pro-apoptotic proteins (36). The anti-apoptotic proteins BCL2 and BCL-XL work to prevent cytochrome c release from mitochondria and, thereby, preserve cell survival. BNIP are cellular proteins targeted by BCL2-related anti-apoptotic factors (45). Both MPM down-regulated a BNIP gene, either BNIP3L for MPM "Sp" or BNIP1 for MPM "Fa". BNIP3 and its homolog, BNIP3L, form a subset of
mitochondrial BH3-containing proteins which cause release of cytochrome c, activation of caspase-3 and apoptotic cell death. BNIP1 is another BNIP pro-apoptotic protein localized in the nuclear envelope. Together they can overcome the suppressor activities of BCL-2, BCL-XL, or E1B antigen and confer additional resistance to apoptosis to MPM. Multiple other DE genes are involved in apoptosis (Table III). They include one overexpressed gene HSPA8 (sp) and five down-regulated genes EPHA2 (co), MFGES (co), PDCD2 (co), UBL1 (co) and REQ (fa), the roles of which should be better appreciated.

The microarray data presented in this work suggest that MPM modulate genes to escape the anti-tumor immune response, although a certain immune recognition may occur. Remarkably, many of those immune resistance genes have already been found by unsupervised analysis that clusters several of them within a so-called immune-related cluster (Figure 1 and ref. 8). As immunotherapy emerged as a new weapon in the arsenal against mesothelioma (46), this precise part of tumor transcriptomes should not be underestimated with regards to the future design of new immunotherapeutic regimens directed against MPM. We can predict, as a corollary to our study, that promising strategies, aimed at enhancing the immune defense against malignant mesothelioma, should be directed towards multiple targets.

Conclusion

In accordance with many literature reports, the data we generated here support a role for immune mechanisms in the pathogenesis of MPM. To our knowledge, our study showed the first extensive analysis of MPM tissues, coupling molecular data sourced from microarray profiling to those obtained by immunohistochemical staining. Our study emphasizes the interesting correlation among "immune signature" and T cells infiltration of the tumor. Whether or not this is translated into clinical improvement is yet to be tested in controlled trials in which transcriptomes should be determined before treatment. Nevertheless, a striking "immune signature" of two MPM was highlighted, which revealed marked modulation of immunoresistance-associated genes, suggesting that MPM represent a protective phenotype against anti-tumor immunity which, perhaps partially, are involved in the bad prognosis of MPM.

By increasing MPM sampling, it would be possible to translate this knowledge into novel ways of slowing disease progression, especially by developing therapies that counteract MPM immunoresistance. Research into understanding the mechanisms governing immune cells infiltration and the immunoresistance of MPM should enable the development of efficient immunotherapy against MPM.

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References


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