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Original Paper

Expression of MHC Molecules and ICAM-1 on Non-small Cell Lung Carcinomas: Association with Early Lymphatic Spread of Tumour Cells

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Early microdissemination of tumour cells determines the prognosis of patients with apparently localised non-small cell lung cancer (NSCLC). Monoclonal antibodies to epithelial antigens can now be used to detect single carcinoma cells present in mesenchymal secondary organs such as bone marrow or lymph nodes. The present study was designed to obtain insights into the potential role of the immune system in lymphatic and haematogenous microdissemination of NSCLC cells. Using immunohistochemical staining of primary NSCLC, we assessed the expression pattern of molecules mediating an efficient cellular immune response, that is, MHC class I and class II antigens and the intercellular adhesion molecule-1 (ICAM-1). All 58 patients evaluated were staged as free of overt metastases by conventional clinico-pathological screening. Isolated tumour cells in bone marrow or lymph nodes were identified with mAb CK2 to cytokeratin component No. 18 and mAb BerEp-4 to glycoproteins of 34 and 39 kd present on epithelial cells, respectively. MHC class I expression on primary tumours was reduced or absent in 6/10 (60.0%) patients with isolated cancer cells in lymph nodes as compared to 6/33 tumours (18.1%) without such tumour cell dissemination ($P = 0.01$). MHC class II molecules on primary tumours were detected in 1/10 (10.0%) patients with micrometastases to regional lymph nodes and in 10/33 (30.3%) patients without such a tumour cell spread. None of the 10 patients with nodal microdissemination expressed ICAM-1 on their primary NSCLC, while such expression was detectable in 12/33 (36.4%) patients without this dissemination ($P = 0.01$). In contrast, the detection of tumour cells in bone marrow was not correlated to the expression of any of these immunoregulatory molecules. Our data suggest that escape caused by deficient expression of MHC class I antigens and ICAM-1 on tumour cells may support homing or survival of disseminated tumour cells in lymphoid tissue.

Key words: MHC molecules, ICAM-1, micrometastases, lung cancer
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INTRODUCTION

THE PROGNOSIS of patients with operable non-small cell lung cancer (NSCLC) is mainly determined by the extent of residual tumour load after surgery. By using a sensitive immunohistochemical assay, we recently demonstrated that prognostically relevant microdissemination of NSCLC cells into regional lymph nodes had already occurred in approximately 15% of the patients staged as N0 by histopathological examination [1]. In another study, early systemic tumour cell spread was assessed by immunocytochemical detection of individual tumour cells in bone marrow, and demonstrated an incidence of more than 20% among patients classified as M0 by current staging procedures [2]. These observations suggest that some primary lung tumours have a high disseminatory potential leading to early dissemination of malignant cells.

In this study, we characterised primary NSCLC with respect to their expression of several important immunoregulatory molecules. MHC Class I molecules are expressed in most tissues (including bronchial epithelium) and act as restriction elements for the recognition of tumour associated antigens by cytotoxic T lymphocytes. The second class of antigen-presenting molecules, MHC Class II molecules, are not constitutionally expressed on normal epithelium, but can be induced by a variety of cytokines. Besides molecules of the MHC complex, the monomeric

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90–114 kd glycoprotein ICAM-1 is important because it facilitates adhesion between tumour cells and lymphocytes via the lymphocyte function associated antigen-1 (LFA-1) [3]. Cytokine-induced expression of ICAM-1 increases the vulnerability of tumour cells to monocyte- and T cell-mediated lysis [4, 5].

There is evidence that loss of MHC Class I expression and the acquisition of MHC Class II expression and ICAM-1 occurs frequently on primary NSCLC [6, 7], but the prognostic significance of these findings remains contentious [6]. In this study, we show that deficient or absent expression of HLA Class I and ICAM-1 on primary NSCLC cells may support lymph node microdissemination of tumour cells, but does not influence a haematogenous spread to bone marrow.

PATIENTS AND METHODS

Tumour specimens from 58 patients with NSCLC were examined. All patients underwent pre-operatively the conventional staging procedures and were found to have operable primary tumours (TNM stage less or equal T_3 N_2 M_0). The median age at the time of surgery was 58 years, ranging from 40 to 77 years. In general, lobectomies or, if necessary, pneumonectomies were performed, followed by a radical lymphadenectomy.

Information on bone marrow micrometastases was available from all 58 patients. Of these patients, 29 were staged as N0 and 29 were staged as N1–2 by conventional histopathology (Table 1).

Information on nodal micrometastases was available in 43 of the patients. Of these patients, 31 were staged as N0 and 12 as N1 by conventional histopathology. In N0 patients, all lymph nodes were screened for micrometastases, in N1 patients only lymph nodes which were judged as negative by routine histopathology were screened. By using immunohistochemistry, nodal micrometastases was detected in 6 of the N0 patients (19.4%) and in 4 of the N1 patients (33.3%).

Monoclonal antibodies

Expression of HLA-A,B,C-antigens on primary specimens was detected using mAb W6/32 (IgG2a, kindly provided by D. Schendel, Institute of Immunology, Munich, Germany), which reacts with a monomorphic epitope of the HLA-A,B,C/β_2m complex [8]. TAL.1B5 (IgG1) was used to stain the 33-kd-chain of the HLA Class II complex (Dako, Hamburg, Germany [9]). ICAM-1 was stained by the PA3.58-14 antibody (IgG2a), which was a gift from J. Johnson (Institute of Immunology, Munich, Germany [10]). Tumour infiltrating leucocytes were detected by antibody T29/33 directed against the common leucocyte antigen T200 (also called CD45, IgG2b, Boehringer Mannheim, Germany). Localisation of the epithelial tumour cells within the specimen was confirmed by the anti-epithelial antibody Ber-Ep4 (IgG1, Dako [11]), directed against two glycopolypeptides of 34 and 49 kd present on the surface and in the cytoplasm of all epithelial cells with only a few exceptions. Ber-Ep4 was also used to detect single tumour cells in lymph nodes [1]. Bone marrow was screened for the presence of epithelial tumour cells by the use of mAb CK2 (IgG1, Boehringer Mannheim) directed against the cytokeratin polypeptide No. 18 antibody [12].

Primary tumour samples and immunohistochemistry

Representative samples of the primary tumour were obtained from fresh surgical specimens and immediately snap frozen in liquid nitrogen. The frozen tissue was stored at −80° until use. For each frozen tumour sample, 10–15 serial cryostat sections of 5 μm thickness were cut and transferred on to glass slides. After air drying for 24 h, slides were stored at −20°. For

Table 1. Expression of HLA Class I, HLA Class II and ICAM-1 in non-small cell lung carcinomas

	No. of patients	Reduced expression of HLA Class I* *n* (%)	Expression of HLA Class II† *n* (%)	Expression of ICAM-1† *n* (%)
Total	58	17 (29.3)	17 (29.3)	18 (31.0)
T stage				
T1–2	41	11 (26.8)	12 (29.3)	14 (34.1)
T3	17	6 (35.3)	5 (29.4)	4 (23.5)
N stage				
N0	29	7 (24.1)	11 (37.9)	9 (31.0)
N1–2	29	10 (34.5)	6 (20.7)	9 (31.0)
Grade‡				
G1–2	29	6 (20.7)	10 (34.5)	10 (34.5)
G3	28	11 (39.3)	7 (25.0)	7 (25.0)
Tumour histology				
Adenocarcinoma	26	6 (23.1)	9 (34.6)	10 (38.5)
Squamous cell carcinoma	24	6 (25.0)	7 (29.2)	5 (20.8)
Adenosquamous carcinoma	4	2 (50.0)	0	2 (50.0)
Large cell carcinoma	4	3 (75.0)	1 (25.0)	1 (25.0)
Age				
≤60	31	9 (29.0)	6 (19.4)	10 (32.3)
>60	27	8 (29.6)	11 (40.7)	8 (29.6)
Sex				
F	13	4 (30.7)	5 (38.5)	3 (23.1)
M	45	13 (28.9)	12 (26.7)	15 (33.3)

* Less than 10% positive tumour cells. † More than 25% positive tumour cells. ‡ Tumour grading was not available in 1 case.

immunohistochemistry, the slides were fixed in acetone for 10 min and air dried for 45 min. Then, the cryostat sections were stained according to the APAAP (alkaline phosphatase anti-alkaline phosphatase) staining procedure described by Cordell and associates [13]. Following rehydration through incubation with Tris-buffered solution (TBS, pH 7.6) for 15 min, AB-serum (pooled serum from donors with blood group AB, diluted 1:10 with phosphate-buffered solution; Biotest, Germany) was applied for 20 min, to block non-specific binding. Subsequently, specimens were incubated for 45 min with the primary antibody in appropriate dilutions. After each incubation step, the slides were washed three times with TBS. The bridging antibody (Dako) was applied for 30 min, followed by incubation with the APAAP complex (Dako) for 30 min. Staining was developed with fast red solution containing 1 mM levamisole for 15 min. Counter staining was performed with Meyer's haemalum solution.

As a negative control for non-specific antibody binding, the primary antibodies were replaced by irrelevant mouse myeloma proteins of identical isotypes (MOPC 21, IgG1; UPC 10, IgG2; Sigma, Deisenhofen, Germany). The cells from the tumour-surrounding stroma and cryostat sections of histopathologically metastases-free lymph nodes served as positive controls for ICAM-1 and HLA molecule expression.

The slides were evaluated, double-blind, by two observers using light microscopy. HLA Class I expression was considered to be lost, if less than 10% of the tumour cells were stained. HLA Class II and ICAM-1 expression was considered to be induced, if more than 25% of the tumour cells expressed the antigen. To exclude false positive evaluation, tumour-infiltrating leucocytes were identified on consecutive tumour sections by an anticommon leucocyte antigen antibody. In approximately 80% of the cases, both observers obtained the same results; the remaining slides were re-evaluated and a consensus decision was made.

Detection of tumour cells in lymph nodes

Nodal micrometastases was assessed by an immunohistochemical assay as described previously, using the monoclonal antibody Ber-EP4 [1]. Briefly, at primary surgery, all resected lymph nodes were halved. One half was embedded in paraffin for histopathological routine staging (HE), and the other half was snap frozen in liquid nitrogen within 3 h after their removal and stored at −80° until use. Lymph nodes which had no evidence of nodal metastases by routine histopathology were screened by immunohistochemistry, using the anti-epithelial mAb Ber-Ep 4 for the detection of micrometastatic tumour cells. Ber-EP-4 (IgG1; Dako) is directed against two glycopolypeptides of 34 and 49 kd present on the surface and in the cytoplasm of all epithelial cells except the superficial layers of squamous epithelia, hepatocytes, and parietal cells [11, 14]. The antibody does not react with mesenchymal tissue, including lymphoid tissue [14] and can also be used on paraffin sections. From each lymph node, 4–6 μm cryostat sections were cut from three different levels and transferred on to glass slides pretreated with 3-triethoxysilyl-propylamin (Merck, Darmstadt, Germany). One section per level was stained with the APAAP technique. Antibody-bound alkaline phosphatase activity was detected with Fast Red TT (Sigma), while endogenous alkaline phosphatase was quenched by addition of levamisole.

Detection of tumour cells in bone marrow

The presence of isolated tumour cells in bone marrow was assessed in 58 patients using our immunocytochemical assay for epithelial cytokeratin, as described previously [2]. Briefly, bone marrow was aspirated from one site of the posterior iliac crest. After density centrifugation through Ficoll–Hypaque (900*g*, 30 min), mononuclear cells from the interface were cytocentrifuged on to glass slides. Routinely, five slides comprising 4×10^5 cells were stained and examined per patient. For immunostaining, the mAb CK2 against the epithelial cytokeratin component 18 was used at a concentration of 2.5 g/ml [12]. The antibody reaction was developed with the APAAP-technique, using New Fuchsin stain for visualisation of antibody-bound phosphatase activity.

Statistical analysis

For statistical analysis contingency tables were tested by Pearsons χ^2 and Fisher's Exact test was used whenever appropriate.

RESULTS

Expression of MHC antigens and ICAM-1 on primary tumour cells

Deficient HLA-A,B,C expression (less than 10% positive tumour cells) was found in 17 of the 58 (29.3%) primary NSCLC carcinomas, while an expression of HLA class II or ICAM-1 was observed in 29.3 and 31.0% of the tumours, respectively (Table 1).

A deficient expression of HLA Class I antigens was observed more frequently in tumours from node-positive patients than in tumours from patients without metastatic lymph node involvement (34.5% versus 24.1%, respectively, Table 1). Furthermore, deficient Class I expression was twice as frequent in undifferentiated (G3) tumours than in well (G1) or moderately (G2) differentiated tumours, but these differences were statistically non-significant.

A comparison of expression of HLA class II or ICAM-1 with conventional clinicopathological risk factors revealed no significant differences (Table 1). However, it should be noted that 10 of the 26 (38.5%) adenocarcinomas were ICAM-1 positive compared to only 5 of the 24 (20.8%) squamous cell carcinomas. Additionally, tumours of patients at a more advanced T stage were found to express ICAM-1 less frequently. 14 of the 41 T1–2 tumours (34.1%) were ICAM-1 positive, whereas T3 tumours expressed ICAM-1 in only 4 of the 17 (23.5%) cases.

Correlation to the presence of disseminated tumour cells in bone marrow

Individual epithelial cells in the bone marrow were detected in 16 of the 58 (27.6%) patients with NSCLC (Table 2). A comparison with clinicopathological parameters revealed that bone marrow micrometastases appeared to be more frequent in patients with T3 tumours, lymph node involvement and undifferentiated tumours (Table 2), but these differences were statistically non-significant.

Comparing the expression of MHC molecules and ICAM-1 in primary tumours from patients with or without tumour cell dissemination to bone marrow, we did not observe any meaningful differences (Table 3). Reduced HLA class I expression or induction of HLA class II antigens or ICAM-1 occurred as frequently in the group of patients with bone marrow micrometastases as in patients without tumour cell spread to the bone marrow.

Correlation with the presence of disseminated tumour cells in lymph nodes

By using immunohistochemistry, isolated tumour cells in lymph nodes, staged as negative by conventional histopathology,

Table 2. Clinicopathological parameters in patients with bone marrow micrometastases

		Patients with bone marrow micrometastases*	
	n	*n* (%)	*P*-value
All patients	58	16 (27.6)	
T stage			
T1–2	41	9 (21.9)	
T3	17	7 (41.2)	0.13
N stage			
N0	29	5 (17.2)	
N1–2	29	11 (37.9)	0.07
Grade†			
G1–2	29	5 (17.2)	
G3	28	11 (39.3)	0.06

* Samples of 4 × 10^5 nucleated cells/aspirate from the upper iliac crest were stained with mAb CK2 using the APAAP technique. † Tumour grade was not available in 1 case.

Table 3. Expression of HLA molecules and ICAM-1 in patients with bone marrow micrometastases

	Patients with bone marrow micrometastases* *n* = 16 (%)	Patients without bone marrow micrometastases *n* = 42 (%)	*P*-value
Reduced HLA Class I expression†	5 (31.3)	12 (28.6)	0.74
Expression of HLA Class II‡	4 (25.0)	13 (31.0)	0.65
Expression of ICAM 1‡	6 (37.5)	12 (28.6)	0.51

* Samples of 4 × 10^5 nucleated cells/aspirate from the upper iliac crest were stained with mAb CK2 using the APAAP technique. † Less than 10% positive tumour cells. ‡ More than 25% positive tumour cells.

were detected in 10 of the 43 patients (23.2%), with no significant differences with respect to various clinicopathological parameters, such as T-stage, N-stage or grading of the tumour (Table 4).

The number of these patients with reduced HLA Class I expression (27.9%) or induced HLA Class II (25.6%) or ICAM-1 (27.9%) expression (Table 5) was comparable with that of the whole cohort (Table 1). Of the 10 patients with nodal micrometastases, 6 displayed reduced HLA Class I expression in their primary tumour, while of the 33 patients with negative lymph nodes, a deficient HLA Class I expression was found in only 6 (18.1%) primary tumour specimens ($P = 0.01$) (Table 5).

Only 1 of the 10 patients (10%) with lymphatic spread of tumour cells was HLA Class II positive compared with 10 of 33 (30.3%) node-negative cases. No patient with lymph node micrometastases was ICAM-1 positive, but 12 of 33 (36.4%) lymph node negative cases were ICAM-1 positive ($P = 0.01$).

DISCUSSION

Alterations in the expression pattern of MHC molecules have been described in many types of solid tumours, including

Table 4. Clinicopathological parameters in patients with lymph node micrometastases

		Patients with nodal micrometastases*	
	n	*n* (%)	*P*-value
All patients	43	10 (23.3)	
T stage			
T1–2	33	7 (21.2)	
T3	10	3 (30.0)	0.56
N stage			
N0	31	6 (19.4)	
N1	12	4 (33.3)	0.33
Grade			
G1–2	27	8 (29.6)	
G3	16	2 (12.5)	0.20

* Lymph nodes which were negative by conventional histopathology were screened by immunohistochemistry with mAb BerEp-4 for disseminated epithelial cells.

NSCLC [6, 15, 16]. In view of its role as a restriction element for cytotoxic T-cell lysis, deficient HLA Class I expression is thought to facilitate the escape of individual tumour cells from the immunosurveillance in the patient. Recently, we were able to demonstrate that reduced HLA Class I expression occurs in approximately 30% of all NSCLC carcinomas, but did not influence the prognosis of the individual patient [6]. Herein, we observed, for the first time, deficient Class I expression in 60% (6/10) of patients with early micrometastatic spread of tumour cells to regional lymph nodes. Although the analysis is limited by the relatively small number of patients, this observation suggests that downregulation of HLA Class I expression on NSCLC might be of biological significance. Moreover, primary tumours from patients with early spread of tumour cells to regional lymph nodes expressed MHC Class II molecules in only 10% (1/10) of the cases, while 30.3% (10/33) patients without isolated tumour cells in lymph nodes had MHC class II expression in their primary tumour. This is consistent with our recent observation of a co-ordinated expression of these molecules in NSCLC [6]. Similar observations have been reported from patients with cervical cancer demonstrating a downregulation of HLA Class I antigens and a lack of MHC Class II expression in regional lymph node metastases [17].

In patients with bone marrow micrometastases, the incidence of HLA Class I negative primary tumours was similar to the overall group of patients, suggesting that the haematogenous spread of lung cancer cells might be independent of HLA Class I expression. Furthermore, the expression pattern of HLA Class II molecules in these patients was comparable to patients without systemic spread of tumour cells. This difference between systemic and nodal microdissemination suggests that lymphatic spread of tumour cells might be more tightly controlled by immune effector mechanisms than haematogenous spread to bone marrow.

While the expression of HLA molecules on primary NSCLC was not found to be of prognostic significance in our recent study [6], we describe herein that downregulation of these molecules is associated with tumour cell microdissemination into regional lymph nodes, which is known to be an independent predictor of a shortened disease-free survival [1]. This discrepancy might be explained by the fact that the patients in our study represent a more homogeneous group, displaying only early stage disease.

Table 5. Expression of HLA molecules and ICAM-1 in patients with lymph node micrometastases

	All patients $n = 43$ (%)	Patients with nodal micrometastases* $n = 10$ (%)	Patients without nodal micrometastases $n = 33$ (%)	P-value
Reduced HLA Class I expression†	12 (27.9)	6 (60.0)	6 (18.1)	0.01
Expression of HLA Class II‡	11 (25.6)	1 (10.0)	10 (30.3)	0.19
Expression of ICAM-1‡	12 (27.9)	0	12 (36.4)	0.01

* Lymph nodes which were negative by conventional histopathology were screened by immunohistochemistry with mAb Ber Ep-4 for disseminated epithelial cells. † Less than 10% positive tumour cells. ‡ More than 25% positive tumour cells.

In a preliminary univariate Kaplan–Meier analysis, we observed a trend that patients with a reduced or absent MHC Class I expression had shortened overall survival ($P = 0.061$; log rank test, data not shown).

The biological and clinical significance of ICAM-1 expression by tumour cells is still under discussion. Cytokine-induced expression of ICAM-1 appears to increase the vulnerability of tumour cells to lysis by non-specific effector cells such as monocytes [4], and ICAM-1 seems to support an effective activation of HLA Class II restricted T cells [5]. Our present study shows that, in patients with ICAM-1 positive primary tumours ($n = 12$) no spread of isolated tumour cells was observed in regional lymph nodes, supporting the view that ICAM-1 molecules facilitate lymphocyte mediated cytotoxicity. Notably, expression of ICAM-1 on human melanoma cells and high levels of circulating ICAM-1 in serum has been associated with increased risk of metastasis and poor clinical outcome [18, 19]. Thus, the prognostic significance of ICAM-1 expression by primary tumour cells seems to depend on the type of tumour.

In conclusion, our data indicate that a deficient or absent expression of molecules mediating a cellular immune response, such as MHC class I antigens and ICAM-1, may favour early lymphatic spread of lung cancer cells, while the development of bone marrow micrometastases appears to be unaffected. This finding suggests that the immune system controls are particularly important for preventing the homing and survival of individual tumour cells disseminated to lymphatic organs.

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