Squamous cell carcinoma: infiltrating monocyte/macrophage subpopulations express functional mature phenotype

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Summary
Biopsies from 26 patients with advanced stage squamous cell carcinoma of the head and neck were investigated to determine the intensity of the inflammatory cellular infiltrate and the expression of leukocyte antigens. Mononuclear cell infiltration varied considerably between the individual patients and also within the tumour. Tumour-infiltrating cells consisted mainly of T lymphocytes and monocytes (Mo/macrophages (MΦ)). Staining procedure with monoclonal antibodies (moabs) against Mo/MΦ revealed different clusters of antigen expression: (1) moabs 27E10 and a-CD35 detected a subgroup of Mo/MΦ with particular staining of perivascular Mo; (2) moab a-CD1 stained preferentially cells in tumour cell clusters; (3) moabs that reacted with cells of either typical MΦ or dendritic morphology throughout the tumour-tissue: a-Fcγ receptor I-III, a-class II antigens, a-CD4, RM3/1, a-CD36 and 25F9. Thus, the majority of tumour-infiltrating mononuclear phagocytes were found to possess a rather mature phenotype. The number of Mo/MΦ with mature phenotype within the tumours correlated with T lymphocyte infiltration in the tissue.

Tumour growth is influenced by cells and/or products of the tumour's microenvironment. These influences are mainly due to cells of the defence system (Boheim et al., 1987; Kopper & Lapis, 1985; Zeromski et al., 1986; Kabawat et al., 1983). In vitro assays have demonstrated a significant role for T cells, natural killer (NK) and lymphokine-activated killer (LAK) cells in killing and lysing of neoplastic cells (Patek & Collins, 1988; Strohme et al., 1987; Whiteside et al., 1988; Vinzenz & Micksche, 1987; Gottlinger et al., 1985). Activated macrophages (MΦ), also, can bind to and destroy neoplastic cells in vitro and in vivo (Fidler & Schroit, 1984; Fidler & Schroit, 1988; Kopper & Lapis, 1985). Furthermore, MΦ, via cytokines, are capable of enhancing lymphocyte-mediated immunity (Bentzen, 1988). On the other hand, depending on their localisation in tissue, MΦ may enhance rather than inhibit tumour growth, possible by triggering local immuno-suppression (Gronberg et al., 1989; Kopper & Lapis, 1985; Yamanaka et al., 1988; Krones, 1988). Considering this bimodal role of MΦ in immunesurveillance of tumours in vitro we focused our studies on the phenotype and surface receptor expression of MΦ, which could be involved in local immunesurveillance of malignant growths.

Patients and methods

Patients

The study group consisted of 28 patients with histologically confirmed diagnosis of a squamous cell carcinoma of the upper gastro-intestinal and respiratory tract with varying degree of differentiation (Table I). All patients were in an advanced stage of their disease, graded T1 and T2, according to the UICC classification of 1987 (Hermanek et al., 1987); 14 patients had evidence of regional metastases, none had distant metastases at the time of surgery. The patients' (25 males, three females) ages ranged from 35 to 71 years (median 56.5). The patients had not received chemotherapy, radiotherapy or previous surgery.

Preparation of tissue sections

Excised tissue blocks, consisting macroscopically of tumour taken from the primary tumour focus, were snap-frozen in liquid-nitrogen cooled methylbutan and stored under liquid nitrogen until use. Serial cryostat sections (6 μm), mounted on glass slides were air-dried for 2 h and fixed at room temperature for 5 min in acetone, then for 5 min in chloroform and finally for 5 min in acetone. The rest of the tumour was fixed in 40% formaldehyde and embedded in paraffin; sections were stained with haematoxilin-eosin for diagnosis and grading at the Department of Pathology of the University of Vienna.

Immunocytochemistry

Source and specificity of monoclonal antibodies (moabs) are listed in Table II. Appropriate dilutions were determined in preliminary experiments. Immunostaining was performed with an indirect peroxidase system as described in detail earlier (Koller et al., 1986).

Double immunostaining

To distinguish between CD4⁺ MΦ and other CD4⁺ cells or between CD16⁺ granulocytes and other CD16⁺ cells, a double immunohistochemical staining was employed. First, moabs

| Table 1 | Patient details |
|---------|----------------|
| Patient | TU localisation | TNM | TU histological grading |
| 1       | oropharynx     | TN₁ | high                  |
| 2       |                | TΝ₂ | high                  |
| 3       |                | TΝ₃ | high                  |
| 4       |                | TΝ₄ | poor                  |
| 5       |                | TΝ₅ | high                  |
| 6       |                | TΝ₆ | high                  |
| 7       |                | TΝ₇ | high                  |
| 8       | hypopharynx    | TΝ₁ | high                  |
| 9       |                | TΝ₂ | moderate              |
| 10      |                | TΝ₃ | poor                  |
| 11      |                | TΝ₄ | high                  |
| 12      |                | TΝ₅ | moderate              |
| 13      |                | TΝ₆ | moderate              |
| 14      |                | TΝ₇ | high                  |
| 15      |                | TΝ₈ | high                  |
| 16      |                | TΝ₉ | poor                  |
| 17      |                | TΝ₁₀| poor-moderate         |
| 18      |                | TΝ₁¹| moderate-high         |
| 19      |                | TΝ₁²| poor                  |
| 20      |                | TΝ₁₃| poor                  |
| 21      | larynx         | TΝ₁₄| high                  |
| 22      |                | TΝ₁₅| high                  |
| 23      |                | TΝ₁₆| moderate              |
| 24      |                | TΝ₁₇| moderate              |
| 25      |                | TΝ₁₈| high                  |
| 26      |                | TΝ₁₉| moderate              |

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Leu4 (a-CD3) or VEP9 (a-CD15) were used as described before and staining procedure continued with the respective second moab (Leu3a = a-CD4 or BW209 = a-CD16) using an alkaline phosphatase/anti-alkaline phosphatase technique (Cordelli et al., 1984). Controls consisted of irrelevant antibodies of the IgG1 (BIP-1) and IgM (SQ4F3) isotypes respectively. Both control moabs reacted with B et 1, the major allergen from birch pollen (Jarolím et al., 1989).

**Evaluation of tumour infiltrating cells**

Cells were counted in five representative areas in a square lattice with 100 squares sized 0.04 × 0.04 mm, final magnification × 400. Positive cells were expressed as absolute numbers of immunostained cells per mm². Statistical correlation was performed by linear regression analysis and as the values were unevenly distributed, a non parametric test was used (Mann–Whitney U test) as a two group test.

**Results**

**Clinical histopathology**

The tumours were graded histologically into 15 cases of highly, five cases of moderately and eight cases of poorly differentiated squamous cell carcinomax. Mononuclear cell infiltration of the tumours was found to vary considerably between the individual patients and also within each tumour.

T cells, defined by a-CD3, a-CD4 and a-CD8 moabs and MΦ, stained by MΦ-markers (Table II) were located predominantly in the tumour stroma. Variable numbers of T cells (58–1017 mm⁻²) and particularly MΦ (197–1397 mm⁻²) were found, some of which had infiltrated also into the tumour cell clusters. Quantitation of tumour-infiltrating cells in 22 patients revealed marked T cell infiltration and concomitantly high numbers of MΦ (Figure 1).

![Figure 1: Leucocyte surface antigen-expression on tumour-infiltrating cells. Columns show median values: high T cell infiltration and strong expression of HLA-DR, HLA-DP, FcyRI (CD64), II (CD32), III (CD16), CD4 and RM3/1 in 22 patients. In contrast, significant fewer MΦ of patients with low T cell infiltration expressed MΦ-antigens characteristic for mature and activated MΦ.](Image)
**Marker for Mo/MΦ subpopulations**

Staining by moabs directed against the three FCy receptors (Figure 2f,g,h), CD4 (Figure 2c), HLA-DR (Figure 4a), HLA-DP and the Rm3/1 (Figure 2e) antigens respectively, was found on most MΦ present in the specimen studied (500–1400 cells mm⁻²). Antibodies of this cluster also correlated significantly with each other (Table III). The number of MΦ expressing complement receptors CR1 (CD35) and CR3 (CD11b) varied considerably, forming thus an intermediate group with respect to antigen expression. Moabs a-CD1, a-CD36, a-HLA-DQ, 25F9 and 27E10 recognised only subgroups of Mo/MΦ and were found on about 25–40% of MΦ, defined by HLA-DR and a-Fcy receptor I–III moabs (Figure 3). Out of these moabs, only a-CD11b correlated significantly with the cluster of moabs mentioned above (Table III). The other moabs (a-CD1, a-CD35, a-CD36, a-HLA-DQ, 25F9, 27E10) formed a different group showing strong correlation between CD36 and CD35 (r = 0.772 P < 0.001). Moabs 27E10 and a-CD36 particularly stained perivascular cells with monocyte morphology (Figure 3b,d). Anti-CD1, which recognises an epitope on Langerhans cells of the skin, in most cases stained only few cells in tumour specimens, which – unlike T cells or other MΦ – were found mainly within solid tumour nests (Figure 3a).

In six patients, only small numbers (58–155 mm⁻²) of CD3⁺ lymphocytes were present in the tumour tissue, and in these cases also the number of MΦ was low (169–502 mm⁻²). The differences in the MΦ-marker expression between these six and the other 22 patients were significant with respect to moabs a-CD4, a-Fcy receptors I, II, III, a-HLA-DR and Rm3/1. Only minor or no significant differences were seen with the other Mo/MΦ moabs 27E10, a-CD1, 25F9, a-CD36, a-CD35, a-HLA-DQ and a-CD11b (Figure 1).

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**Figure 2** Immunostaining on cryostat sections of tumour tissue with monoclonal markers directed against leucocyte surface antigens. a–h, × 244.4, interference contrast, counterstained with nuclear fast red. a, Section stained with a-CD3 (T lymphocytes). b, Adjacent section stained with a-CD8. c, Area similar to that in a and b stained with a-CD4. d, Cells stained with a-CD11b (CR3). e, Cells stained with moab Rm3/1. Cells stained with a-CD64 (FcγRI, f), a-CD32 (FcγRII, g) and a-CD16 (FcγRIII, h). Note the dendritic morphology found of positive cells in e–h (>).
Table III Correlations of antigen expression

| moabs | CD4-MΦ | CD64 | CD32 | CD16 | CD3 |
|-------|--------|------|------|------|-----|
| CD4-MΦ | 1      | 0.584** | 0.640** | 0.793*** | 0.718*** |
| CD64   | 0.584** | 1    | 0.696*** | 0.696*** | 0.507*  |
| CD32   | 0.640** | 0.883*** | 1     | 0.803*** | 0.414*  |
| CD16   | 0.793*** | 0.696*** | 0.803*** | 1     | 0.538** |
| CD3    | 0.718*** | 0.507*  | 0.414*  | 0.538** | 1     |
| HLA-DR | 0.438*  | 0.518*  | 0.520*  | 0.511** | 0.383* |
| HLA-DP | 0.383*  | 0.534*  | 0.615** | 0.591** | 0.437* |
| Rm3/1  | 0.202ns | 0.771*** | 0.665*** | 0.479* | 0.113ns |
| CD1    | 0.576** | 0.286ns | 0.452*  | 0.602** | 0.185ns |
| 25F9   | 0.346ns | 0.244ns | 0.158ns | 0.404*  | 0.070ns |
| CD11b  | 0.310ns | 0.228ns | 0.292ns | 0.468*  | 0.303ns |

***P<0.001; **P<0.01; *P<0.05; n.s. = not significant.

Figure 3 Distribution of Mo/MΦ markers in tumour tissue. a–e, Cryostat sections, × 249.6, interference contrast, counterstained with nuclear fast red. a, Cells stained with a-CD1 (Langerhans cells of the skin). Positive cells are found rather in the solid tumour than in the stroma. b, Section stained with moab 27E10 (subpopulation monocytes). Only few cells with monocyte-like appearance are labelled. c, Similarly, only few cells are detected by a-CD35 (CR1), similar in shape to that seen in b. d, Cells stained with a-CD36 (Mo/MΦ). Note the perivascular position of positive cells as found in b (>). e, 25F9+ cells (mature MΦ).

Figure 4 Expression of Mo/MΦ markers on tumour cells. a–c, × 234, interference contrast, counterstained with nuclear fast red. a, Cells with MΦ-morphology (>) but also tumour cells (→) stain positive with a-HLA-DR. b, Tumour cells show reactivity with 27E10 (Mo). c, Tumour cells, forming a horny pearl, stain positively for a-CD36 (Mo/MΦ) (→). In the upper left corner a CD36+ MΦ.
 Independently from the histological grading of the whole tumour, in 11 cases the tumour cells expressed class II antigens (Figure 4a). In 13 specimens, tumour cells forming non-differentiated parts - like horny pearls or stratified epithelium - reacted with moab 27E10 and a-CD36 (Figure 4b,c).

Low numbers of B cells (CD22+ cells) and granulocytes (CD15+ cells) were detected in the tissues studied, the latter cells being found predominantly in necrotic areas (data not shown).

Discussion

The highly divergent results on the numbers of tumour infiltrating Mo/MΦ may be due to the fact that these cells mainly express functional antigens, present on the surface of these cells only during certain steps of activation. Characterisation of cultured blood Mo showed that at the beginning of culture about 60% express the 27E10-antigen (Zwadlo et al., 1986) and will lose it in favour of the antigens Rm3/1 and later 25F9 (Zwadlo et al., 1985; 1987). Other studies pointed out that cultured blood Mo gained or showed enhanced expression of Fcy-receptors (Bougammart et al., 1988; Clarkson & Ory, 1988), HLA-DR (Peters et al., 1987) or the CD4 antigen (Crowe et al., 1987). The results of our study indicate that most MΦ found in tumour tissues are of a functionally 'mature' phenotype. It is not surprising that in our study the numbers of tumour infiltrating these cells correlated with a high number of infiltrating T cells, as T lymphocyte products may account for migration of mononuclear phagocytes and further enhance their differentiation towards mature effector cells (Burchett et al., 1988; Makovsky et al., 1988).

It is certainly of interest to compare the tumour infiltrate seen in squamous cell carcinomas with the infiltrate found in tumours and inflammatory tissues of other origin. Other authors found that in malignant melanomas and gastric carcinomas 27E10+ and 25F9+ macrophages were associated with tumour progression (Bröcker et al., 1987; Heidt et al., 1987; Bröcker et al., 1988). In acute gingival inflammation the dominant Mo/MΦ population carried the 27E10 antigen, whereas the numbers of Rm3/1 and 25F9 positive cells were low (Zwadlo et al., 1985, 1986, 1987). Furthermore, double staining revealed that all three markers labelled distinct, non overlapping MΦ subpopulations. In contrast, in chronic inflammatory processes high numbers of Rm3/1 and 25F9 positive cells were found, some of these cells carrying both antigens simultaneously (Zwadlo et al., 1987). Our present results with the tumour tissue thus resembles the pattern of Mo/MΦ infiltration found in chronic inflammatory lesions. In spite of the marked inflammatory reaction within the tumours of most patients, we could not correlate the number of T lymphocytes with histological grading or tumour differentiation. This finding makes a major influence of tumour malignancy on the number and composition of inflammatory infiltrates unlikely, although subtle differences may have escaped detection because of the limited number of patients and tumour samples investigated. Furthermore, functional defects of immunocompetent cells or the lack of specific immunological responses against putative tumour antigens may explain the ineffectivity of the infiltration to destroy the tumour tissue.

Thus, functional properties of tumour infiltrating cells have to be characterised in vitro and in situ in more detail, by excluding a crucial defect in cytokine and cytotoxic production. This appears to be especially important, since trials are already performed using immune response modifiers or specific anti-tumour antibodies in the treatment of malignancies (Ozawa et al., 1989; Mace et al., 1988). The finding that squamous cell carcinoma cells may express MΦ markers (as MHC class II antigens) has been recently described by Russel et al. (1988), who found the expression of PGP-1, which is strongly expressed by mouse phagocytes, also on human bladder carcinoma cells when these cells were transplanted into nude mice. These data suggest that these MΦ antigens could be induced by mediators of the defence system to generate additional molecules for intercellular adhesion. It seems further likely that squamous carcinoma cells only with a certain differentiation-grade would be able to express these antigens.

In our study, it is shown that the tumour-infiltarting Mo/MΦ population is equipped with surface antigens necessary for enhanced cellular interaction and tumour cell killing. These MΦ could eventually act as specific effector cells, e.g. when a monoclonal antibody is added - which in turn gives them an important role in future immunotherapy.

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