PROFILE OF IMMUNE CELLS IN LYMPH NODES DRAINING HUMAN MALIGNANT TUMORS

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Abstract

The purpose of this study was to characterize and quantify cells involved in immune response in metastasis-free regional lymph nodes (RLNs) draining different human epithelial tumors and compare them (by immunohistochemistry) with control lymph nodes from patients with non malignant diseases. We showed that T cells number was decreased in RLNs as compared to the controls with reduction in both CD4+ T cells and CD8+ T cells subsets and an inverted ratio (CD4+: CD8+). B lymphocytes and follicular dendritic cells were decreased with respect to the controls. S100+ dendritic cells (DCs) and mature DCs were detected in T dependent areas. Their mean number was significantly lower as compared to control. Immature DCs were significantly diminished compared to RLN and control nodes. CD57+ cells, follicular T helper cells and/or NK cells, were localized in the clear zone of germinal centres and their mean number was significantly increased. There were no CD57+ cells in hypoplastic follicles. In this study we show that RLNs draining human cancer present reduction in almost all immune cells, except CD57+ cells. These findings may be related to the deficient anti-tumor immune response in patients with cancer and subsequent tumor progression.

Key words: lymph nodes, cancer, immune cells

Regional lymph nodes (RLNs) draining malignant human tumors, one of the first components of the human immune system to have contact with tumor cells or their products, are considered the first barrier against tumor progression1. The most decisive evidence of the existence of an immune response to tumors in humans, are the histological changes in RLNs, consisting of an prominent sinus histiocytosis and hyperplastic germinal follicular centres, even in absence of metastatic tumor cells. These reactions would represent a host response against tumor cells in which all the immune cells are involved2. The cellular composition and the highly specialized architecture of lymph nodes facilitate the interaction of antigen presenting cells (APC) with lymphocytes resulting in a specific immune response. In order to carry out this response efficiently, tumor antigens must be recognized, processed and presented by specific cells so that different lymphocyte subsets can be activated, soluble factors secreted and memory lymphocytes generated. Dendritic cells (DCs) are considered the most efficient APC capable of initiating and maintaining an anti-tumor specific immune response.

Received: 21-IV-2008 
Accepted: 4-IX-2008

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Resumen Perfil de las células inmunes en los ganglios linfáticos que drenan tumores malignos humanos. El objetivo del trabajo fue caracterizar y cuantificar utilizando inmunohistoquímica, las células involucradas en la respuesta inmune en ganglios linfáticos regionales (GLRs) que drenan distintos tumores epiteliales malignos humanos y compararlas con ganglios controles (GLCs) provenientes de pacientes sin enfermedad neoplásica maligna. Determinamos que los GLRs presentaban una marcada depleción de linfocitos B y T, células dendríticas (CD) foliculares y CD interdigitantes maduras respecto a los controles. En los linfocitos T, además de estar disminuidos, se observó una inversión de la relación T CD4+: T CD8+, a favor de los T CD8+. La depleción de CD inmaduras fue mayor respecto a las maduras. Las células CD57+, células foliculares T helper y/o células NK, localizadas en las zonas claras de los centros germinativos, presentaron un marcado incremento en los GLRs comparados con los GLCs, excepto en los casos de ganglios linfáticos con folliculos hipoplásicos. En este estudio, demostramos que los GLRs que drenan carcinomas humanos presentan una significativa reducción en casi todas las células de la respuesta inmune, excepto las células NK. Estos hallazgos podrían estar relacionados con la deficiente respuesta antitumoral de los pacientes con cáncer y la subsiguiente progresión tumoral.

Palabras clave: ganglios linfáticos, células inmunes, cáncer
These cells reside in resting or "immature" state in non lymphoid tissues, where they efficiently capture and process antigens. In response to different microenvironment stimuli, DCs maturate up-regulating both MHC and costimulatory molecules on their surface. They migrate as veiled cells to lymph nodes and become potent antigen presenting cells as interdigitating dendritic cells, where they trigger naïve T cells. After recognizing the antigen, CD4+ T helper cells are activated, releasing cytokines that activate CD8+ T cytolytic cells whose enzymes (granymes, perforins) can lead to tumor cell death by apoptosis. The mannose CD1a is another molecule expressed on DCs surface and often used as DC marker. Structurally it is related to the MHC molecules and can present non-peptide antigens such as lipids and glycolipids to T cells.

For a long time B cells, the main components of the lymphatic follicles, were considered unimportant in the anti-tumor immune response. However, recent studies have shown that B cells when activated, up-regulate MHC and co-stimulatory molecules initiating an antigen specific anti-tumor response, priming CD8+ T cytotoxic cells or acting as antigen presenting cells.

Germinal centers (GC) of lymphatic follicles are dynamic structures with a dark zone where B cells proliferate and a clear zone where their selection and differentiation occur. This is the zone where follicular dendritic cells (FDC) and CD4+ T helper cells form an intricate network, immune complexes are trapped and recognized by B cells. Selected B cells would either proliferate or differentiate into plasma cells and memory B cells. Those cells not doing that, die via apoptosis.

Antibodies to CD57 epitope (HNK-1) have been used to identify a T cell type in GC in human tonsils, spleen and lymph nodes, follicular helper T cells (TFH). Recent studies have demonstrated that these cells present characteristics of T helper CD4+. CD57+ T helper cells of GC, express the chemokine receptor CXCR5, a pattern consistent with their specific location. They constitute an important fraction of resident CD4+ T cells, and it has been proposed that CD57+ cells are a novel effector T cell subset different from other well known effector T cell subsets such as Th1 and Th2 cells. Kim et al. demonstrated that these cells are highly efficient in helping B cell production of all subsets of immunoglobulins. Due to their specific localization in GC, the activities of CD57+ GC T helper cells on B cell proliferation and antibody production have been studied by several groups of researchers.

Histological modifications in RLNs draining tumors are particularly relevant in cancer and suggest that these changes reflect some functional and anatomical disturbance probably influenced by tumor-associated factors. Based on these findings, the aim of this study was to characterize and quantify the immune cells in regional lymph nodes without metastasis, draining different types of epithelial cancer and compare them to control lymph nodes (LNs) of patients without malignancies.

**Materials and Methods**

**Patients:** Regional lymph nodes from 86 patients with different malignant tumors were collected. Their histological types and origins are shown in Table 1. Tumor stages were first classified according to TNM pathologic assessment in agreement with the TNM International Classification.

The absence of metastasis in lymph nodes was determined by routine histological methods. Draining lymph nodes presenting metastasis were not included in this study. It should be emphasized that none of the patients received chemotherapy, immunotherapy or radiotherapy before the study, and none of them presented evidence of local or systemic infections. Because the study of human lymph nodes from healthy individuals is limited by ethical considerations, we used 26 lymph nodes from patients (aged 35 and 75 years) who underwent surgery for usual non malignant diseases (20 from cholelitiasis and 6 from colonic diverticulosis). The methods performed did not have influence on the diagnostic and therapeutic procedures for patients and there was no conflict with the ethical requirements of the Bioethics Committee of our hospital.

**TABLE 1.** - Histological types and origins of malignant tumors

<table>
<thead>
<tr>
<th>Squamous cell carcinoma</th>
<th>Adenocarcinoma</th>
<th>Transitional carcinoma</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larynx n= 19</td>
<td>Breast n=37</td>
<td>Bladder n= 2</td>
<td>Thymoma (Thymic carcinoma) n=1</td>
</tr>
<tr>
<td>Lung n=11</td>
<td>Colon n=2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esophagus n=3</td>
<td>Parotid n=1</td>
<td></td>
<td></td>
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<tr>
<td>Cervix n=2</td>
<td>Thyroid n= 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral mucosa n=1</td>
<td>Stomach n= 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin n=1</td>
<td>Ampulla of vater n=1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lips n= 2</td>
<td>Gallbladder n=1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total= 86</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Tissue blocks of regional lymph nodes from cancer and control patients were studied as follows: after excision, the lymph nodes were fixed in 10% buffered formalin for paraffin embedding. Simultaneously half of the lymph node was snap-frozen and cryostat sections (4-5 mm thick) were obtained for immunohistochemical staining with monoclonal antibodies applicable only on frozen sections.

Immunohistochemistry: The primary antibodies used in this study were polyclonal antibody S-100 (DakoPatts), monoclonal antibodies (mAB) CD1a (DakoPatts Ab, Stockholm (Sweden), CD 86 (DakoPatts Ab, Stockholm, Sweden), CD8 (DakoPatts Ab, Stockholm, Sweden), CD45-RO (Immunotech SAS, Marseille, France), CD22 (Immunotech SAS, Marseille, France), CD35 (DakoPatts Ab, Stockholm, Sweden) and CD57 (DakoPatts Ab, Stockholm, Sweden).

Five micrometers sections from the formalin-fixed, paraffin-embedded tissue blocks were obtained, mounted on slides coated with 3-aminopropyltriethoxy-silane (Sigma Diagnostics, St. Louis, MO, USA), deparaffinized with xylene, passed through graded alcohol, and rinsed three times in deionised water and phosphate buffered saline (PBS), pH 7.2. Pressure cooker pre-treatment was performed. In all cases, sections were pre-incubated with 3% hydrogen peroxide in water to block endogenous peroxidase activity. Slides were incubated for one hour at room temperature with specific monoclonal antibodies: CD22 and CD45 RO with the purpose of identifying B and T memory cells, respectively. To detect FDC and interdigitating DCs, mAB CD35 and polyclonal S-100 was used respectively and to detect CD 57+ germinal cells, CD57 was used.

Frozen sections were employed to detect cytotoxic T cells and helper T cells with mAB CD8 and mAB CD4 and to identify immature and mature DCs, CD1a and CD86 were used respectively.

After washing the primary antibody three times with PBS, the slides were incubated with a biotinylated link antibody and finally with the streptavidin-peroxidase complex. The avidin-biotin-peroxidase complex (Vectastain ABC KIT, Peterborough, England) method was used for immunohistochemical staining and Carbazol chromogen for visualization. Finally, sections were counterstained with Mayer Haematoxylin (BioPack, MR, Buenos Aires, Argentina) and mounted with glycerol-vinyl aqueous solution (Zymed Lab. Inc. San Francisco, USA).

Computer Image Analysis: Images of each sector (follicles centers and paracortical zone) were obtained. To avoid duplications, the observation was made in a greek embroidery fashion. Histological images were captured from a microscope (Olympus BX50 system microscope, Tokyo, Japan) with an objective magnification of x40, through a video camera attached (Sony DXC 151A CCD colour video camera, Tokyo, Japan). The images thus obtained were digitized with a 24-bit true colour TIFF format (Pentium II, 266 MHz, 64Mb RAM, FlashPoint 128, (Integral Technologies, Inc., Indianapolis, IN, USA), software: Image-Pro Plus for Windows v3.01 (Media Cybernetics, Silver Spring, MA, USA). The grid matrix of the images was calibrated as to give a yield of 0.32 mm/pixel.

To separate the immunostaining (red stain) from the haematoxylin staining (blue stain) the cube-based method of the colour segmentation operation was applied. The threshold value was set with a sensitivity of 4 (maximum 5). A mask was then applied in order to make the separation of colours permanent. The images were then transformed into an 8-bit grey scale TIFF format. The number of stained cell was then counted. Data values obtained from at least 10 images of each slide were exported to a spreadsheet in order to perform the statistical analysis.

Statistical analysis: comparisons between the cancer group and the control group were analysed by the alternate Student T test. All statistical tests were considered significant at the p<0.05 level.

Results

The histological study of regional lymph nodes draining cancer showed some changes in the architecture and in lymphoid cells types. Most RLNs without histological evidence of metastases showed follicular germinal center hyperplasia and sinus histiocytosis. Follicular germinal center hyperplasia was a prominent change in RLNs draining squamous cell carcinoma from larynx, lung, esophagus, lips and skin. In contrast, in RLNs draining adenocarcinoma from gastrointestinal tract, parotid, thyroid or breast, sinus hyperplasia was the main architectural change and in some of them (10%) the presence of hypoplastic follicles was observed. All lymph nodes used as controls displayed different levels of reactive changes without acute lymphadenitis, reflecting their dynamic structure.

The analysis of lymphoid cells in RLNs obtained from patients with different types of cancer was performed to determine their number and phenotype profile.

T lymphocytes, identified as CD45 RO+ cells (904 ± 304), were significantly decreased in RLNs as compared with control lymph nodes (1253 ± 987). A reduction in both T CD4+ cells (139 ± 56 ) and in T CD8+ cells (595 ± 132) with respect to control nodes (6659 ± 845 and 1571 ± 175, respectively) was observed and additionally there was an inverted ratio (CD4+:CD8+) between those T cell subsets (normal, 2:1 ratio). All these cells were anatomically located in the paracortical T dependant areas. B lymphocytes, identified as CD22+ cells (214± 34 ) were significantly lower with respect to control lymph nodes (4340 ± 679) (Table 2). They located mainly in the cortical follicles, specially in the outer rim surrounding the germinal centers.

S-100 protein, a marker expressed by several types of dendritic cells, was detected in cells with their typical morphology in T-dependent areas. Their mean number was lower in RLNs (2490 ± 251) as compared to lymph nodes (4576±156). In germinal centers, the FDC population identified with CD35 antibody was diminished (969 ± 124) in relation to control lymph nodes (2920 ± 304), were significantly decreased in RLNs as compared to control nodes (6659 ± 845 and 1571 ± 175, respectively) was observed and additionally there was an inverted ratio (CD4+:CD8+) between those T cell subsets (normal, 2:1 ratio). All these cells were anatomically located in the paracortical T dependant areas. B lymphocytes, identified as CD22+ cells (214± 34 ) were significantly lower with respect to control lymph nodes (4340 ± 679) (Table 2). They located mainly in the cortical follicles, specially in the outer rim surrounding the germinal centers.

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In RLNs, CD86+ DCs were detected in T dependent areas and exhibited similar morphology to S100+ DCs. Their mean number (2510 ± 255) was similar to S100+ DCs and diminished as compared to control (3292 ± 506). CD1a+ DCs subset was lower (458 ± 100) as compared to control (2519 ± 518) (Table 4).

CD57 was used to assess germinal center T cells. In all the cases, CD57+ cells were localized in the clear zone.
of germinal centres. Their mean number (68 ± 4) was significantly increased as compared to control lymph nodes (23 ± 8) in concordance with follicular germinal center hyperplasia (Table 5). In lymph nodes with hypoplastic follicles, CD57+ cells were absent.

**Discussion**

Our results showed a decrease of cells involved in the cellular immune response in metastases-free RLNs draining malignant tumors. Ignorance or tolerance to tumor antigens by reactive T cells in both central and peripheral lymph organs constitute the basis of the loss of immunosurveillance. The mechanism by which the immunosupression is produced in the lymph nodes is complex and poorly understood. The development of an immune response against the tumor depends on the DCs capability of presenting tumor antigens in the RLNs where a specific tumor response is induced. Its presence in the peritumoral infiltrate suggests the existence of a close relationship between the host immune system and the tumor.

Our results indicate that in RLNs without metastases there is a significant decrease of DCs as compared to control lymph nodes. This reduction may be attributed to the fact that the tumor cells may induce dendritic cell apoptosis in the peritumoral infiltrate as observed in tumors of aggressive behaviour. The decrease of expression co-stimulatory molecules in DCs population in RLNs could also explain one of the mechanisms of local immunosupression.

The number of FDCs located at the germinal centers of the RLNs was notably reduced, and this fact may impair B cell proliferation.

Quantitative analysis of T cells showed marked reduction and inverse CD4+:CD8+ cells ratio with respect to control lymph nodes (normal, 2:1 ratio). However, this imbalance towards cytotoxic CD8+ T cells is not translated into an efficient antitumor immune response possibly due to inhibitory factors released by tumor cells. It was shown that the FAS-L expression in breast carcinoma produces an increased ability of tumor cells to induce apoptosis in T cells.

Germinel center CD57+ cells were strikingly increased. Adequate evidence indicates that CD 57+ cells predominantly located in the follicular germinal center light zone, are CD4+ cells. They arrive to GC following a chemokine gradient. These cells, namely TFH or GC Th cells efficiently provide B cell help in peripheral lymphoid organs. These cells are a critical component of the GC response; in the absence of T cell help, functional GCs are not formed. Despite their central role in humoral immunity, little is known about the Th populations in GC. There are contradictory reports regarding the B cell helping function of CD 57+ cells in production of immunoglobulin.
It is still unknown the reason by which malignant cells growth in immunocompetent hosts. It is known that malignant tumors can express diverse antigens. In spite of this, an efficient immune response for preventing tumor growth has not been developed. The depletion of mature and immature DCs, the quantitative and qualitative alteration of T cells and B cells contribute to the development of tolerance in RLNs and create a microenvironment favorable for tumor growth and metastases development. Reversal of tumor-induced immune suppression by administrating cytokines or bioreagents to modulate the tumor or the draining lymph node microenvironment, would be an interesting approach to the management of solid malignancies.

References


Experience is the great teacher: unfortunately, experience leaves mental scars, and scar tissues contract.

La experiencia es la gran maestra, infortunadamente, la experiencia deja cicatrices mentales y las cicatrices se contraen.

William J. Mayo (1861-1939)