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Comparative analysis of CD1a, S-100, CD83, and CD11c human dendritic cells in normal, premalignant, and malignant tissues

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Summary. A number of antibodies that recognize human dendritic cells (DC) have been identified. The main aim of this study was to compare and contrast different antigen retrieval techniques using both enzymatic and non-enzymatic treatments in order to determine the expression and distribution of several DC markers on formalin-fixed, paraffin-embedded tissues. Normal human lung, oral epithelial hyperplasia lesions, oral squamous cell carcinoma, and prostate adenocarcinoma tissues were evaluated using a panel of DC specific antibodies. The results of immunohistochemical staining for CD83, CD1a, CD11c, and S-100 DC markers were compared following the different antigen retrieval approaches. The overall best results for the analysis of tumor-associated DC were obtained with the enzymatic methods. Protease XXIV digestion was determined to be essential for detection of S-100 and CD11c positive DC, whereas trypsin and pepsin were required for the recognition of CD1a and CD83 expressing tumor-associated DC. These results could be easily adapted for routine practice and should be useful for characterization of the DC system in cancer patients for both diagnostic and prognostic purposes. In addition, standardized procedures for evaluating different subpopulations of tumor-associated DC should bring new insights in understanding of DC-tumor cell interaction.

Key words: Dendritic cells, Oral cancer, Prostate cancer, Immunohistochemistry

Introduction

Dendritic cells (DC) perform an essential role in the generation and regulation of both innate and acquired immune responses, including antitumor immunity. Immature DC are widely distributed throughout the body and occupy sentinel positions in non-lymphoid tissues. They constantly sample their environment for antigens by phagocytosis, macropinocytosis, and highly efficient receptor-mediated endocytosis. In the presence of appropriate inflammatory signals, immature DC undergo maturation characterized by the up-regulation of surface MHC and costimulatory molecules and rearrangement of chemokine receptors. Subsequent migration to the lymphoid tissues results in efficient presentation of optimally processed antigens to T lymphocytes.

The DC system, thus, is a major contributor to host immunity and immune surveillance against malignancy. Therefore tumors might develop mechanisms suppressing the activity of DC in order to evade immune recognition and elimination. In fact, other research as well as our own has recently demonstrated that different tumors inhibit DC generation, maturation, function, and survival (Oyama et al., 1998; Pirtskhalaishvili et al., 2000; Aalamian et al., 2001; Katsenelson et al., 2001; Shurin et al., 2001a, 2002). These data demonstrate that rapidly growing tumors are usually poorly infiltrated by DC and unable to trigger the recruitment and activation of DC resulting in delayed or insufficient antitumor immune responses. However, the mechanisms regulating DC migration to and homing within the tumor are poorly understood.

Analysis of tumor-associated DC (TADC) was initially focused on the immunohistochemical and histological evaluation of DC localization and distribution in the tumor tissues using CD1a and S-100 antigens as markers for TADC. Later, additional DC-related markers CD83 and p55 became available to characterize DC within different tissues. The most important result of these studies was the suggestion that the outcome of a primary tumor in cancer patients depends on the level of tumor infiltration by DC (Becker, 1992; Austin, 1993; Nestle et al., 1997). For instance, immunohistochemical analysis of tumor

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infiltrating DC revealed that an increased number of DC within tumors correlates with a better prognosis in colorectal adenocarcinoma (Ambe et al., 1989), and gastric, esophageal, and nasopharyngeal carcinomas (Nomori et al., 1986; Tsujitani et al., 1990; Imai and Yamakawa, 1993). Patients with higher levels of TADC demonstrate a longer survival and lower metastases (Becker, 1992, 1993). For example, S-100-positive DC within the malignant tumors or around them were studied by Ambe et al. (1989) in one hundred and twenty-one patients with colorectal adenocarcinoma. Five-year survival rate was 70.5% in patients with high numbers of S-100+ DC and 33.3% in patients with a few S-100+ cells (Ambe et al., 1989). Furthermore, DC are not recruited in large numbers into renal cell carcinomas (Troy et al., 1998b), prostate cancer (Troy et al., 1998a), breast carcinoma (Bell et al., 1999), and metastatic melanoma (Garcia-Plata et al., 1995) in advance stages correlating with poor prognosis. Thus, these data suggest that (i) human tumors with a high DC infiltrate develop no or low levels of metastases, (ii) the presence of DC in tumor biopsies correlates with the survival of cancer patients, and (iii) the absence of DC in many tumors is associated with poor prognosis (Shurin and Gabrilovich, 2001). Thus, the number of DC in the tumor tissue could be considered as a marker of tumor grade, tumor aggressiveness and progression, and possibly might serve as a criterion of the status of the DC system in cancer patients. However, the further development of immunodiagnostic tests based on the number of TADC, as well as the assessment of immunobiology of DC within the tumor, is limited by undeveloped and non-standardized immunohisto-chemical techniques for the growing number of DC markers and by incomplete knowledge of the biological significance of these markers.

A few monoclonal antibodies that recognize human DC have been applied in routine immunohistochemical analysis of formalin-fixed paraffin specimens for experimental and diagnostic purposes (Okuyama et al., 1998; Chen et al., 2000; Dallal et al., 2002; Pileri et al., 2002). Nevertheless, immunohistochemical staining of non-fixed frozen samples mounted in OCT compound is the most common method for the characterization of new TADC markers in clinical research laboratories (Ikeguchi et al., 1998; Bell et al., 1999; Troy et al., 1999; Maeda et al., 2002; Iwamoto et al., 2003), although it is frequently associated with poor cytological details (Falini et al., 1989; Pileri et al., 1997). In contrast, known problems of paraffin-embedded specimens are difficulties identifying specific antigens due to the masking effect of fixatives, e.g. formalin-induced cross-linking of proteins (Huang, 1975; Pileri et al., 1980, 1997; Ezaki, 2000). To overcome the limitations in antigen accessibility in formalin-fixed samples, proteolitic enzymes could be used for tissue pretreatment. It has been shown that precise timing, careful temperature control, and specific enzyme concentrations are key factors required for breaking the bonds produced by fixation and uncovering a higher number of antigenic epitops (Huang, 1975; Reading, 1977; Shintaku and Said, 1987). For instance, heat-induced antigen retrieval in a variety of solutions using a Bunsen burner, microwave oven, pressure cooker, or autoclave all have been shown to enhance the immunoreactivity of a wide range of antigens in routine formalin-fixed paraffin-embedded tissues (Shi et al., 1991; Cattoretti et al., 1992; Kawai et al., 1994; Pileri et al., 1997). However, simultaneous analysis of different DC markers in normal and malignant paraffin-embedded human tissues, as well as determination of the most appropriate antigen retrieval technique for each antigen, has not been yet described. Standardized procedures for evaluating different subpopulations of TADC should bring new insights in understanding of DC-tumor cell interaction and serve as a basis for developing new diagnostic and prognostic tools in cancer.

The main aim of this study was to compare and contrast different antigen retrieval techniques, using both enzymatic and non-enzymatic treatments, in order to determine the expression and distribution of several DC markers on formalin-fixed, paraffin-embedded tissues. Normal human lung, oral premalignant hyperplasia lesions, oral squamous cell carcinoma, and prostate adenocarcinoma tissues were evaluated using a panel of antibodies of diagnostic and experimental relevance. The results of immunohistochemical staining for CD83, CD1a, CD11c, and S-100 DC markers were compared following the different antigen retrieval approaches. The overall best results for the analysis of TADC were obtained with the enzymatic methods. Protease XXIV digestion was determined to be essential for detection of S-100 and CD11c positive DC, whereas trypsin and pepsin were required for the recognition of CD1a and CD83 expressing TADC. These results could be straightforwardly adapted for the routine practice and should be useful for characterization of the DC system in cancer patients for both diagnostic and prognostic purposes.

Materials and methods

Tissues

Immunohistochemical studies were performed on a variety of formalin-fixed and paraffin-embedded tissue sections, including normal human lung, spleen, oral epithelial hyperplasia lesions (5 blocks), oral squamous cell carcinoma (5 blocks), and prostate adenocarcinoma (20 blocks) specimens. Non-fixed frozen sections of normal human lung mounted in OCT compounds were used for comparative analysis. A panel of tissues was selected on the basis of anticipated positive or negative reactivity with antibodies to each antigen tested in this study. All tissue sections were selected from the Tissue Bank of the Department of Pathology, University of Pittsburgh Medical Center. Four-micrometer sections were cut, mounted on positively charged slides

("superfrost/plus", Fisher Scientific, Chicago, IL) and allowed to dry overnight at 56°C in order to ensure optimal adhesion. Before immunohistochemistry, sections were dewaxed in xylene, rehydrated through a series of graded ethanol solutions, and rinsed in phosphate-buffered saline (PBS), followed by different antigen retrieval procedures.

Antigen retrieval (AR)

DC specific antibodies were applied to tissue sections, which had undergone the following AR procedures: (i) Digestion with 1 mg/ml trypsin (Sigma, St. Louis, MO) at 37°C for 20 min; (ii) Digestion with 0.05% pepsin solution (Sigma), pH 2 at 37°C for 20 min; (iii) Digestion with 0.05% protease XXIV solution at 37°C for 5 min. After the proteolytic treatments, the sections were placed in a cold buffer (4°C) for 10 min, in order to block the activity of the enzyme. (iv) Microwave treatment in 0.01 M citrate buffer solution (Na citrate/citric acid) (pH 6). Rehydrated sections were immersed in citrate buffer solution and processed in a microwave oven (Spacemaker III, GE, Menlo Park, NJ) with the power set at high (power 10, 90°C) for 4 min, then at power set at 4 for two cycles (15 min each). After completion of microwave treatment, sections were allowed to cool at room temperature for 20 min, and then rinsed in PBS.

Antibodies and immunohistochemical procedures

Mouse anti-human monoclonal antibodies were used as primary antibodies to detect DC localization in tissue sections. Monoclonal antibodies recognizing CD83 and CD1a (Immunotech, Westbrook, ME), CD11c (DAKO, Carpinteria, CA), and S-100 (Sigma) proteins were included. Mouse anti-human CD68 antibodies (DAKO) were used for detection of macrophages. The working dilutions of primary antibodies were determined in preliminary titration experiments performed with human spleen or oral hyperplasia lesions. Each type of antibody was tested in five different conditions: 1) without AR of the sections, 2) with AR using a microwave, 3) with AR using digestion with trypsin, 4) with pepsin, and 5) with protease XXIV solutions.

Immunohistochemical staining was performed using avidin-biotin peroxidase (ABC) technique. After AR procedures, tissue sections were treated with 3% hydrogen peroxide to inactivate endogenous peroxidase. The sections were then washed with three changes of PBS and treated with normal 10% horse serum for 10 min to block non-specific binding. Appropriately diluted mouse anti-human antibodies against CD83 (1:50), CD1a (non diluted), CD11c (1:100), S-100 proteins (1:1000), and CD68 (1:50) were applied to each section for 60 min (with exception for anti-CD1a antibodies, which were applied overnight) at room temperature. As a control, inappropriate antibodies of the same isotype were used instead of primary antibody. After washing with PBS, the slides were incubated with biotinylated horse anti-mouse IgG (H+L) antibody (1:250, Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature and then with the avidin-biotin-complex ABC (avidin DH:biotinylated horseradish peroxidase H complex, Vector Laboratories). The catalyzed amplification procedure using tyramide (NEN, Boston, MA) was applied for detection of CD1a and CD11c positive cells. HRP reaction was developed at room temperature in a solution of amino-9-ethylcarbasole (AEC, Scytec, Logan, UT). After counterstaining with hematoxylin, slides were mounted with permount (Fisher Scientific, Fair Lawn, NJ) for light microscopic observation. All slides were read independently by two investigators.

Results

Establishment of the appropriate retrieval technique for the detection of DC markers in formalin-fixed paraffin-embedded normal human spleen and oral epithelial hyperplasia lesions

Table 1 summarizes the findings obtained with the CD1a, S-100, CD83, CD11c, and CD68 antibodies employed. A series of titration were performed for each antibody, aiming to assess optimal working dilution with each AR system. Table I includes the optimal dilution for all antibodies used. To work out the optimal AR procedure for the detection of CD11c antigen, we used formalin-fixed paraffin-embedded normal human spleen

Table 1. Comparative analysis of different antigen retrieval procedures with antibodies most commonly used for the detection of human dendritic cells.

ANTIBODY (SOURCE)	ANTIBODY DILUTION	NO AR	TRYPSIN, 1 mg/ml	PROTEASE XXIV, 0.05%	PEPSIN, 0.05%	MICROWAVE 0.01 M citrate bufer
CD1a (Immunotech)	Undiluted	_	+	_	_	_
S-100(Sigma)	1:1000	_	_	+	_	_
CD83 (Immunotech)	1:50	_	_	_	+	_
CD11c(DAKO)	1:100	_	_	+	_	_
CD68(DAKO)	1:50	_	_	_	_	+

AR, antigen retrieval; "-", no detectable staining; "+", positive immunostaining.

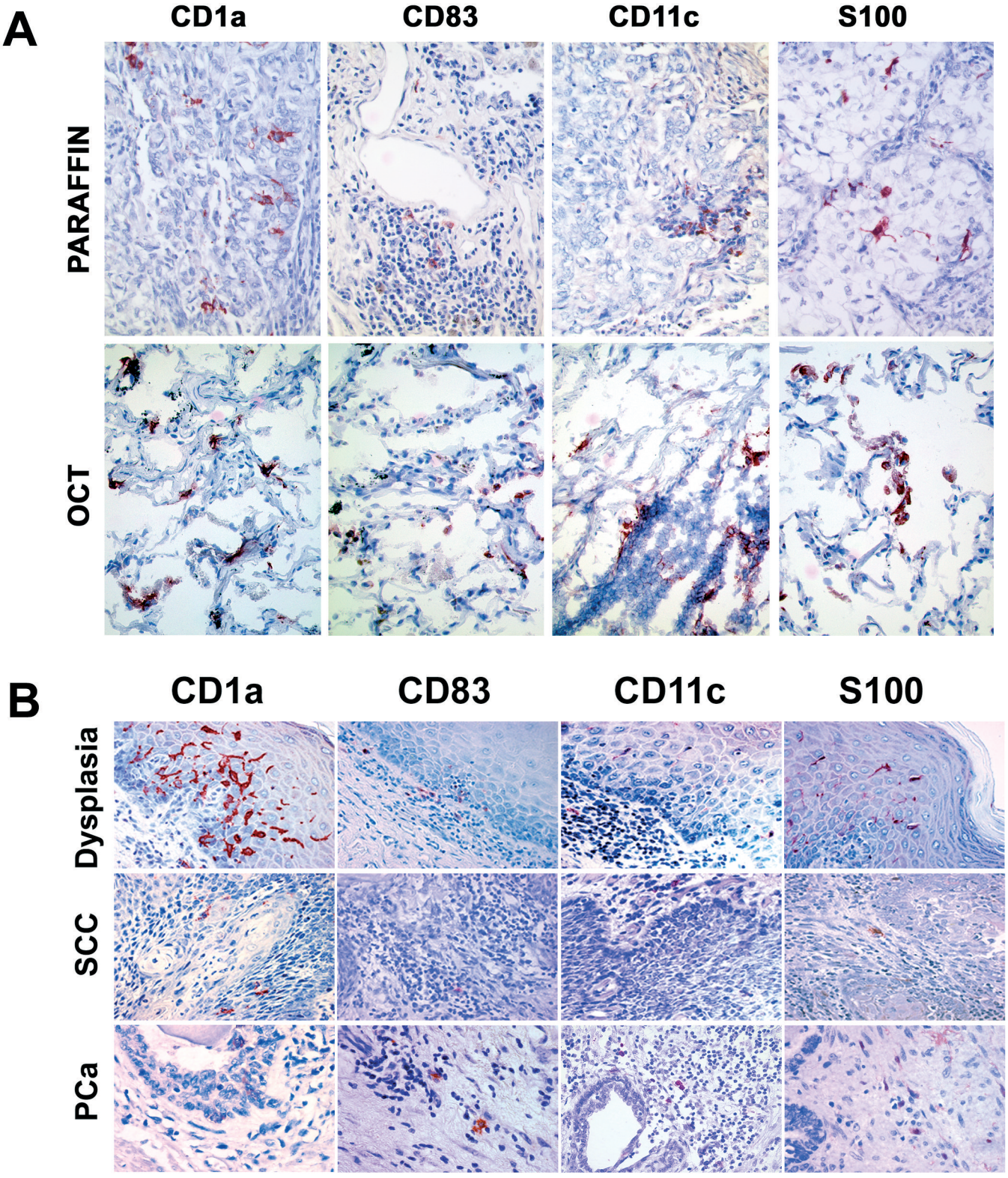

Fig. 1. A. Immunohistochemical staining of CD1a, S-100, CD83, and CD11c positive DC in the formalin-fixed paraffin embedded and non-fixed OCT frozen normal human lung tissues. Comparative analysis. **B.** Representative staining pattrn of CD1a, S-100, CD83, and CD11c positive DC in formalin-fixed paraffin-embedded human oral hyperplasia lesions, squamous ccell carcinoma, and prostate adenocarcinoma.

due to a known high level of CD11c expression (McIlroy et al., 2001). The spleen has been also used for detection of macrophages (CD68). CD1a and S-100 positive cells are known to represent Langerhans cells and are found mostly in the basal and parabasal layers in squamous epithelia (Pileri et al., 2002; Zavala et al., 2002). Glycoprotein CD83 is one of the best-known maturation markers for human DC and primary expressed on activated DC (Lechmann et al., 2002a). The results shown in Table 1 demonstrate that different AR procedures are required for the detection of CD1a, S-100, CD11c, and CD83 positive DC in formalin-fixed paraffin-embedded sections of oral hyperplasia lesions, characterized by a high level of inflammation, and normal human spleen. Importantly, no DC were detected in formalin-fixed tissue sections without an appropriate antigen unmasking technique.

Furthermore, heat-based AR was suitable only for the anti-CD68 antibody, which allowed staining of tissue macrophages. Proteolytic enzymes were effective in retrieving CD1a, S-100, CD83, and CD11c immunoreactivity in the tissues, although with different specificities. Trypsin was highly effective for the staining of CD1a positive DC (Table 1 and Fig. 1). CD11c and S-100 positive cell were visualized in the tissue after pretreatment with protease XXIV (Table I and Fig. 1). Finally, the anti-CD83 antibody recognized the tissue antigen only after the pretreatment with a pepsin (Table 1. Fig. 1). Thus, the best results for the detection of the DC markers in formalin-fixed inflammatory tissues were achieved when the enzymatic tissue pretreatment procedures were applied. Different DC-related antigens were selectively visualized using specific proteolytic enzyme-based tissue treatment: detection of S-100 and CD11c DC markers requires protease XXIV, while trypsin and pepsin were essential for the detection of CD1a and CD83 positive DC, respectively.

Immunohistochemical staining of normal human lung, oral epithelial hyperplasia lesions, and neoplastic formalin-fixed, paraffin-embedded tissues with DC antigens using optimized AR technique

In the first set of experiments, we compared immunohistochemical staining of CD83, CD1a, CD11c, and S-100 DC markers in both normal human lung formalin-fixed paraffin-embedded and non-fixed frozen samples mounted in OCT compound. We demonstrated that staining of cryopreserved and formalin-fixed tissues for the DC related markers, using appropriate antibody titration and optimal antigen retrieval methods, gave the comparable results (Fig. 1A), suggesting that both immunohistochemical technique could be used for DC analysis in human tissues. However, the staining of cryopreserved tissues demonstrated poor cytology and morphology details.

In the next set of experiments, infiltration of oral hyperplasia lesions, and different tumors by CD1a, S-100, CD83, and CD11c positive DC was evaluated using immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections. Immunohistochemistry was performed using optimized AR technique, which was described above as being effective for each corresponding antibody. The results of these experiments are presented in Fig. 1B. As can be seen, CD1a and S-100 positive DC were observed in high numbers in the premalignant oral hyperplasia lesions and to a different extent in normal human lung (Fig. 1A), squamous cell carcinoma and prostate adenocarcinoma tissues. CD11c positive DC moderately infiltrated lung, and to a lesser extend hyperplasia lesions, prostate adenocarcinoma, and tissues adjunct to the oral squamous cell carcinoma mass. The appearance of high levels of CD83 positive DC was demonstrated in the hyperplasia lesions, moderate level in normal human lung, but not in the prostate adenocarcinoma, squamous cell carcinoma. In conclusion, these results demonstrate that inflammatory process in the tissue is associated with a high level of infiltration by different subtypes or subpopulations of DC. Tumor tissue is characterized by a low level of DC infiltration, which might correlate with histopathological grade of the tumor and stage of the disease.

Discussion

DC perform a central role in the initiation and regulation of immune responses, including antitumor immunity, justifying a great interest in DC-based immunotherapies (Shurin et al., 2001b; Buchler et al., 2003; Mosca et al., 2003; Schuler et al., 2003). Infiltration of tumors by DC reflects the host immune defense mechanism and has been associated with better prognosis, reduced tumor recurrence, and fewer metastases (Troy et al., 1999; Chen et al., 2000; Kikuchi et al., 2002; Iwamoto et al., 2003). However, in spite of a great number of identification of TADC in different tumors (for review see (Shurin and Gabrilovich, 2001; Shurin et al., 2003)), there is still a lack of understanding of TADC function and correlation between different DC subpopulations within the tumor mass and tumor progression. One of the reasons for these limitations is the absence of developed and standardized protocols of immunohistological determination of TADC in formalin-fixed tissues using constantly appearing new DC markers. For instance, CD11c positive DC have never been described in formalin-fixed tumor tissues due to the absence of immunohistochemical protocols. In addition, there are only a few reports simultaneously utilizing 2 or 3 DC-recognizing antibodies in the same tissue, which significantly limits our knowledge of the functional significance of different DC subtypes in cancer.

In this study, a panel of antibodies recognizing human DC was applied for immunolabeling of paraffin sections of normal lung, oral hyperplasia lesions (site of inflammation and premalignant abrasion), oral squamous cell carcinoma, and prostate adenocarcinoma tissues using different antigen retrieval techniques. AR methods

included digestion with proteases (1 mg/ml trypsin, 0.05% pepsin solution, 0.05% protease XXIV solution) and heating in citric acid buffer to unmask different cross-linked epitops. Several antibodies, including CD1a, S-100, CD83, and CD11c, were found to recognize tissue DC when combined with an appropriate AR procedure.

Historically, CD1a positive DC were first identified in epithelial surfaces as Langerhans cells. However, later it was demonstrated that CD34+ hematopoietic precursor-derived DC, as well as monocyte-derived DC, also express CD1a molecules in *in vitro* cultures. It is generally accepted now that CD1a is a marker of immature human DC (Troy et al., 1999; Zavala et al., 2002; Iwamoto et al., 2003). Expression of S-100 protein on human DC was reported more than 30 years ago, serving for a long time as the only marker for tumor-infiltrating DC. S-100 protein was discovered in the bovine brain and was introduced as a specific protein to nervous tissue (Moore, 1972). Later on it was demonstrated that Langerhans cells of the skin, interdigitating cells of lymph nodes, and some cells in the lymphoreticular tissues are positive for S-100 protein. At present, S-100 protein is known to be a distinguishing marker of human DC and is widely used for evaluating TADC in primary and metastatic tumors. Both CD1a and S-100 positive TADC were found to correlate with patients' survival (Tsuge et al., 2000; Reichert et al., 2001; Dallal et al., 2002).

CD83 is one of the well-known maturation markers for human DC, since it was first detected on human blood DC by Zhou and Tedder (1995). CD83 has been shown to be expressed on activated and mature human DC and is markedly up-regulated together with co-stimulatory molecules CD80 and CD86 during DC maturation. Interestingly, the first clear proof that CD83 is important for DC biology came from recently performed studies using a soluble form of the extracellular CD83 domain: DC-mediated T cell proliferation could be completely inhibited using this recombinant molecule (Lechmann et al., 2002a). Furthermore, CD83 -/- knockout mice revealed a block in CD4+ T cell generation, suggesting a new possible immunomodulatory function of CD83 (Lechmann et al., 2002b). These results are in agreement with data demonstrating that mature activated DC are essential for the recruitment and survival of activated tumor-specific lymphocytes during carcinogenesis (Chen et al., 2000; Dallal et al., 2002; Shimizu et al., 2002; Iwamoto et al., 2003). Thus, CD1a, S-100, and CD83 currently serve as acceptable DC markers for immunohistochemical evaluation in human tissues. Here for the first time, immunohistochemical determination of human DC in formalin-fixed tissue simultaneously using these three DC markers was described.

Surprisingly, there are no reports evaluating human CD11c positive DC infiltration tumors and their potential role in antitumor immunity. In mice, CD11c is an established marker for most murine DC populations with the exception of CD11c low plasmacytoid DC subset. In humans CD11c+ DC have been found in the peripheral and cord blood, thymus, tonsils, and spleen (Dalloul, 2001; McIlroy et al., 2001; Summers et al., 2001; Osugi et al., 2002). The precise role of CD11c+ DC in the regulation of immune responses and particularly in the generation of antitumor immunity has not been yet determined. Lissoni et. al have demonstrated that the percentage of cells expressing CD11c molecules in peripheral blood of cancer patients was significantly lower than in healthy volunteers. Furthermore, the level of CD11c positive DC was significantly lower in patients with metastatic disease than in patients with no metastases (Lissoni et al., 1999). These findings suggest that CD11c+ DC might represent a new biological marker predicting a poor prognosis in human neoplasm. However, CD11c+ DC subsets have not been fully evaluated in human tumor tissues. Here we described, for the first time, immunohistochemical method, which allowed measuring CD11c+ DC in formalin-fixed paraffin-embedded sections of normal human lung, spleen, oral hyperplasia lesions, oral squamous cell carcinoma, and prostate adenocarcinoma tissues.

Using an appropriate AR technique, we found that oral hyperplasia lesions are highly infiltrated by S-100 and CD1a expressing DC, and to a lesser extent by CD11c and CD83 positive DC. CD11c positive DC were observed in human lung, in some cases of prostate adenocarcinoma, but were mainly absent in squamous cell carcinoma tissues. Similarly, few or no CD1a-positive DC and minimal or no expression of the DC activation markers CMRF-44 and CMRF-56 were revealed in human breast cancers (Coventry et al., 2002). On the contrary, evaluation of DC infiltration in primary and metastatic colorectal tumors using immunohistochemistry for the DC marker CD83 and the DC activation molecules CD40 and CD86 revealed that nearly all CD83+ cells were also CD40+ and CD86+, indicating that the DC that infiltrate colon cancer in vivo express the activation and costimulatory molecules associated with a mature DC phenotype (Schwaab et al., 2001). Furthermore, the density of DC in colorectal cancer primaries was three times lower than that seen in normal colonic mucosa, and DC density in metastases was six-fold lower than in colorectal primary tumors. Our data showed that in early stages of tumor progression, squamous cell carcinoma and prostate adenocarcinoma were characterized by the presence of S-100 and CD1a expressing DC, while CD83 positive DC were usually absent.

In summary, our results highlight the opportunity for standardized and reproducible identification of CD1a, S100, CD83, and CD11c expressing DC in formalin-fixed paraffin-embedded human tissues using an appropriate antigen retrieval technique. As demonstrated in direct comparison assays, different DC-specific antibodies exhibit different sensitivity to AR procedures. Protease XXIV digestion was found to be necessary for

detection of S-100 and CD11c DC markers, while trypsin and pepsin were required for uncovering CD1a and CD83 expression, respectively. These data provide an experimental basis and tools for systematic evaluation of different DC subpopulations in patients with cancer for both diagnostic and prognostic purposes.

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