Alveolar stem cells in canine bronchial carcinogenesis

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Abstract

Alveolar type II cells are not present in normal epithelium of canine segmental bronchi but after carcinogen exposure they do occur in intra-epithelial lesions with all degrees of atypia and in invasive lesions with different glandular growth patterns. Immunohistochemistry for proliferation markers (PCNA; Ki-67) strongly suggest that such novel type II cells are pluripotential stem cells in canine bronchial carcinogenesis. Very likely, bronchial carcinogenesis is subject to an oncofetal mechanism of differentiation: bronchial epithelial retrodifferentiation followed by novel differentiation of alveolar tumor stem cells.

Keywords: Bronchial carcinogenesis; Stem (progenitor) cells; Alveolar type II cells; Proliferating cell nuclear antigen; Non-small cell lung cancer; Breast/prostate carcinoma

1. Introduction

Information about stem cells for bronchogenic carcinoma has remained inconclusive. Possible candidates are bronchial epithelial cells that are capable of division [1,2]. Previous studies in our canine model of bronchial carcinogenesis (see below) suggest that the alveolar type II cell may be another candidate [3–5]. Such cells occur in major subsets of bronchogenic carcinoma in humans [6–9] and animals (reviewed in [4]). Type II cells are actively proliferating pluripotential stem cells in normal mammalian lung morphogenesis [10–12]. They display highly distinguishing features, including an approximately cuboid shape, a large and roundish nucleus, cytoplasmic staining for SP-A, and presence of precursory or mature forms of multilamellar bodies [10–14]. In this report we demonstrate that type II cells may play a role as pluripotential stem cells in bronchial carcinogenesis. However, as will be discussed, further evidence is required to prove this conclusion incontrovertibly.

2. Definitions

As defined [3–6], the term ‘cell of origin’ refers to the very first undifferentiated (primordial-like) tumor progenitor cell that appears during transformation (via metaplasia) of normal bronchial epithelium to bronchogenic carcinoma. We use the term ‘stem cell’
to indicate the predominant proliferating cell type that occupies the dividing layers of the (pre)neoplastic lesions.

3. Materials and methods

Canine subcutaneous bronchial autografts (SBAs) are obtained from the bronchial tree of the left lung following pneumonectomy; the major bronchi are cut into segments and placed subcutaneously onto the back through two short midline incisions (10–14 SBAs per dog). Approximately 6–8 weeks later, 3-methylcholanthrene in sustained release implants was placed in the SBAs. As reported [15,16], carcinomas developed in 77% (86/111) of these autografts; metastases occurred in two dogs. Tumors were successfully serially transplanted in athymic nude mice. The bronchial mucosa of each SBA in which there was ongoing carcinogenesis was sampled repeatedly and serially. The intra-epithelial and invasive lesions described here were excised from the SBAs after 6–12 and 16–24 months exposure to carcinogen, respectively.

After fixation in formalin and embedding in paraffin, serial sections were cut at 6 μm and placed on poly-L-lysine coated slides. Adjacent sections were used for immunohistochemistry and hematoxylin and eosin (H&E) staining, which allowed for morphologic characterization of immunoreactive cells and histological typing of the lesions [17,18]. For immunohistochemistry, we used antibodies to: (1) proliferating cell nuclear antigen (PCNA), clone PC10 (Signet Laboratories, Dedham, MA) and Ki-67 proliferating cell antigen, clone MIB-1 (AMAC Inc., Westbrook, ME), mouse anti-human; and (2) natural and recombinant human SP-A, rabbit or mouse anti-human (our antibody SALS-Hu [19,20]; gifts from BYK Gulden Pharmaceuticals, Konstanz, Germany [21] and other investigators). SALS-Hu was prepared in rabbits as reported [19]; the SP-A specificity was assessed by immunohistochemistry, Western blotting, and in vitro translation of mRNA from human lungs followed by immunoprecipitation [19,20]. SALS-Hu cross-reacts with SP-A in normal dog lung and canine adenocarcinoma as has been demonstrated by immunohistochemistry [3–5] and Western blotting [3]. The PCNA and Ki-67 antibodies were applied to tissue sections according to the ABC-peroxidase method using biotinylated horse anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA), streptavidin peroxidase (Dako Corp., Carpinteria, CA), and 3,3-diaminobenzidine as the chromogen [6,13]. Prior to incubation, endogenous peroxidase activity was quenched with a freshly prepared 3% hydrogen peroxide solution in methanol. Sections of each specimen were processed for antigen retrieval by microwave exposure in 10 mM citrate buffer (pH 6.0) [22]; for PCNA detection, adjacent untreated sections were used as well. The optimal antibody dilution in phosphate-buffered saline (PBS; pH 7.6) ( Sigma Chemical, St. Louis, MO) was assessed using positive normal tissue controls (colon; lymph nodes). The Ki-67 antibodies were used at a 1:50 dilution, and the PCNA antibodies at a dilution of 1:1000 for microwave treated sections and of 1:200 for untreated sections. The antibodies to SP-A were applied according to the indirect immunofluorescence technique [3–5] and the avidin/biotin complex (ABC) method as above using swine anti-rabbit or horse anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA) [6,13]. The sections were counterstained with Mayer’s hematoxylin.

Immunohistochemical controls were performed on the same tumor material with antibodies to Clara Cell 10 kDa protein (CC10) (rabbit anti-human; gift of Dr. G. Singh, Pittsburg, PA), preimmunization serum (PS) or normal mouse serum as the primary antibody or omission of one of the incubation steps, and on normal canine lung tissues.

4. Results

Lesions that had arisen from the existing normal bronchial epithelium of SBAs after carcinogen exposure showed abnormal epithelial areas with mild to severe atypia and in situ carcinoma, sometimes interspersed with (almost) normal epithelium, and micro- and macro-invasive adenocarcinomas. The intra-epithelial lesion illustrated (Fig. 1A; H&E staining) had two different areas: one area, which is designated here by the term ‘dysplastic focus’ (arrowheads), had basal hyperplasia and squamous metaplasia with mild to moderate atypia, whereas the other area had regular hyperplastic epithelium (on the right). Immunohistochemistry (Fig. 1B) revealed that both areas contained PCNA positive nuclei. This staining pat-
tern was not influenced by the methods used (antigen retrieval versus no antigen retrieval). A similar distribution pattern was found for Ki-67. Closer examination in the H&E-stained section (Fig. 1C) showed that the dysplastic focus had two major categories of epithelial cells. The approximately cuboid cells, which occupied the basal and adjacent layers, had a large nucleus and little cytoplasm. The nuclei were
round to ovoid or sometimes irregular, lightly to moderately or sometimes strongly basophilic, and contained one or sometimes two prominent nucleoli. The second category of cells, which occupied the upper adluminal layers, often had more cytoplasm. Their hyperchromatic nuclei were still rather large, indicating that there was no true squamous maturation in the dysplastic focus. Nuclear PCNA (Fig. 1D) and Ki-67 staining predominated in the basal and one or two adjacent epithelial layers of the dysplastic focus, where mitotic figures were also seen. This indicated that the cuboid cells with a large nucleus and little cytoplasm, which occupied these layers (see Fig. 1C), were the major proliferating cells of the dysplastic focus. As shown (Fig. 1E), such cuboid cells were SP-A positive, whereas the cells in the regular hyperplastic area (on the right) did not stain. The SP-A staining of the cuboid cells was delicate and included the peripheral cytoplasm as also seen in embryonic lungs [12], which suggests cellular immaturity in the lesions [3,4]. A weak SP-A staining was sometimes found in the larger cells present in upper layers of the dysplastic focus. Some staining found along the luminal surface was probably caused by the presence of SP-A which had been secreted into the lesion’s lumen. However, as shown in immunohistochemical controls (see Fig. 1F), it could also be non-specific staining of necrotic material present on the luminal surface. Immunohistochemistry for CC10 provided a positive staining of non-ciliated bronchiolar epithelial cells in normal canine lung but in pre-neoplastic lesions (Fig. 1F), neither cuboid nor other...
epithelial cells displayed any staining. Other immunohistochemical controls were also negative.

A similar relationship between cell type (i.e. cuboid cell with a large nucleus and little cytoplasm) and specific protein (PCNA/Ki-67; SP-A) expressions as found in the dysplastic lesion above was observed in other intra-epithelial lesions with mild to severe atypia, in in-situ carcinoma, and in in-situ and macro-invasive lesions from adenocarcinomas with different (bronchiole-alveolar; acinar; and other) glandular growth patterns.

Fig. 2 gives an example of our findings in adenocarcinomas. The cuboid cells, which occupied the basal and adjacent epithelial layers of the lesions (Fig. 2A; H&E staining), had a large nucleus and little cytoplasm. The nuclei were round to ovoid or sometimes irregular, moderately to strongly basophilic and contained one or sometimes two nucleoli. Occasionally, such cuboid cells were also found locally in more upper (adluminal) epithelial layers. Larger cells with more abundant cytoplasm (Fig. 2A) were present in upper epithelial layers as far as the lumen, alone or in clusters, but never in basal epithelial layers. Their nuclei were lightly to moderately basophilic and roundish and often had one or sometimes two very prominent nucleoli. Nuclear staining for PCNA (Fig. 2B) or Ki-67 usually predominated in the basal and adjacent epithelial layers of the lesions. In a number of lesions, the nuclear staining was present in more upper layers as well. As appeared from the adjacent H&E-stained sections, such variant staining patterns were probably caused by a tangential cut of the lesions. The findings indicated that the basally located cuboid cells with a large nucleus and little cytoplasm were the major proliferating cells of the lesions. As shown (Fig. 2C), all cuboid cells of the basal epithelial layer and some cuboid cells in upper layers stained for SP-A, whereas the larger cells in the upper region were (almost) negative. Staining for CC10 (Fig. 2D) and other immunohistochemical controls were completely negative.

5. Discussion

The data in this paper strongly suggest that alveolar type II cells are pluripotential stem cells in canine bronchial carcinogenesis (see Section 2). Such cells are the predominant proliferating cells of progressive lesions which had arisen from canine SBAs after carcinogen exposure, including intra-epithelial lesions with all degrees of atypia and invasive lesions with different (bronchiole-alveolar; acinar; and other) glandular growth patterns. The type II stem cells usually predominate in the basal region of the lesions near the connective tissue but they may sometimes occur in the upper region as well (present study) [3-5], which suggests that proliferation takes place in both peripheral and luminal directions. However, as shown, these cells may also differentiate to apparently viable larger cells with more abundant cytoplasm that is (almost) devoid of reactivity to SP-A. Such larger cells occur exclusively in upper layers of the lesions.

Light and electron microscopy and immunohistochemistry (present study) [3-5] demonstrate that alveolar type II cells involved in canine bronchial carcinogenesis lack any similarity to other dividing cells in the conducting airways such as mucous and basal cells and the columnar secretory cell type in normal distal bronchioles described by Clara [23]. The tumor type II cells have all the distinguishing features of
their normal counterparts, namely the approximately cuboid cell shape, the large and roundish nucleus, cytoplasmic staining for SP-A, and presence of precursory or mature forms of multilamellar bodies [10–14]. Often, however, the cells look somewhat immature at the ultrastructural level [3] (see Fig. 3). In this respect, they resemble type II cells present in early fetal lungs [11,19] and in distal bronchioles of adult lungs, the latter of which have been incorrectly considered to be Clara cells [12,13].

Type II tumor stem cells first appear in epithelium of SBAs that is slightly abnormal (i.e. basal hyperplasia and squamous metaplasia with mild or moderate atypia) [3,4]. In the embryo, type II cells originate from undifferentiated primordial epithelium but never from earmarked bronchial epithelium [10]. Evidence that undifferentiated primordial cells exist in the epithelium of larger airways after birth is not available [10]. In view of these data and present insights into embryonic lung differentiation [10–12], we have postulated [4] that neoplastic progression for adenocarcinoma development in SBAs may start with local retrodifferentiation of existing normal bronchial epithelial cells such as mucous or basal cells, which results in the appearance of more or less undifferentiated (primordial-like) cells of origin. Differentiation of type II tumor stem cells from such cells of origin may occur by activation of genes that regulate type II cell expression but were repressed in utero in earmarked bronchial cells, a phenomenon not unlike the appearance of alpha fetoprotein in liver cancer. In view of their way of induction (i.e. by carcinogen exposure) and depending on other (growth, genetic, environmental) factors, type II tumor stem cells highly probably give rise to type II cell clones that display significant variations in growth and differentiation potentials.

In normal mammalian lung morphogenesis, type II cells have an enormous growth potential. They generate the entire alveolar epithelial portion of the lung and also maintain it in adulthood [10–12]. In canine bronchial carcinogenesis type II cells give rise to an active yet stable progeny with an apparently high growth and invasive potential (present study) [3–5]. Very likely, the rates of proliferation, differentiation, and slough of type II tumor stem cells and their descendants are responsible for the final size of the lumen and the overall appearance of the adenocarcinomatous lesions. That such cells likely belong to different clones (see above) may contribute to the histologic complexity and diversity of adenocarcinomas. The present study does not exclude that also unrelated clones, e.g. those produced by potential tumor stem cells like mucous and basal cells, could contribute to such pattern formation, although there is no evidence for this possibility at this very moment. Our conclusion regarding a pluripotential stem role of type II cells in canine bronchial carcinogenesis is in agreement with our findings of type II cell populations in serial tumor transplants in nude mice [3,5] and with our and other’s findings of type II cells and SP-A protein or mRNA in bronchiolo-alveolar and other adenocarcinomas in humans and animals [4,6–9]. Further characterization of the stem cell clones involved in canine bronchial carcinogenesis is required to prove our conclusion incontrovertibly.

Our concept that the central duct system of the lung may give rise to peripheral (alveolar) adenocarcinomas due to an oncofetal mechanism of differentiation (epithelial retrodifferentiation followed by novel differentiation) is novel and may be of interest to carcinogenesis studies in other organs with a prominent duct system, e.g. liver, pancreas, prostate, mammary gland, and salivary glands. If our concept is applicable, the duct system of, for instance, the liver may play a more important role in carcinogenesis than has been expected from histologic typing of carcinomas (mostly liver cell tumors). This may especially hold true for breast and prostate carcinomas, in view of the impressive morphogenetic potential of the duct systems in breast and prostate in postnatal life (generation of the alveoli) which may enhance disregulation. Further support for our concept may come from studies of the regulation of epithelial expression in the duct system [24], especially in oncological animal models where lesions are induced at selected focal sites [25].

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