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Neovascularization in hyperplastic, metaplastic and potentially preneoplastic lesions of the bronchial mucosa

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Abstract Angiogenesis is important in a large number of normal and pathological processes including tumour growth and development, inflammation and in wound healing. We investigated whether neovascularization exists in hyperplastic, metaplastic and potentially preneoplastic lesions of the bronchial mucosa as prestages for lung cancer. Biopsy specimens from 86 patients were investigated light microscopically. Formalin-fixed and paraffin-embedded specimens of regular bronchial mucosa including epithelium, basement membrane zone and tunica propria (n = 12) without inflammation were compared with specimens with inflammatory reaction (n = 9), basal cell- and goblet cell hyperplasia (n = 24), squamous cell metaplasia (n = 9), squamous cell metaplasia with different degrees of dysplasia (n = 11), specimens of micropapillomatosis (n = 9) and 13 cases with carcinoma in situ. The grade of neovascularization was assessed by the microvessel density, which was obtained by an immunohistochemical staining of endothelial cells using factor VIII-related antigen and determined by an automatic image-analysing-system. Microvessels were counted in selected areas of highest neovascularization on a × 100 field 0.4 mm underneath the basement membrane zone in the tunica propria. Microvessel count, minimal and maximal diameter of the vessels were chosen as morphological variables. A significantly increased microvessel count with 33 vessels/0.6 mm^2 was found in specimens with inflammation of the tunica mucosa (regular bronchial mucosa: 20 vessels/0.6 mm^2). Microvessel diameter (surface of cut section) increased in specimens of bronchial mucosa with inflammation to 11.3 × 10^{-4} mm^2 (regular bronchial mucosa: 9.04 × 10^{-4} mm^2). Microvessel count increased in cases of squamous cell metaplasia (33 vessels/0.6 mm^2) squamous cell metaplasia with different degrees of dysplasia (50 vessels/0.6 mm^2) and carcinoma in situ with 61 vessels/0.6 mm^2. With increasing dysplasia, increasing neovascularization was found in close vicinity to the basement membrane zone. Simultaneously, interepithelial sprouts of endothelial cells were seen. Qualitative and quantitative differences were thus found in potentially preneoplastic lesions.

Key words Bronchial preneoplastic lesions · Neovascularization · Factor VIII labelling

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Introduction

The reaction of the bronchial mucosa to acute and chronic injuries is manifest either in transitory regenerative activities, or in more permanent changes such as loss of epithelial differentiation or the onset of intraepithelial neoplastic transformation. Reparative processes include basal cell hyperplasia, goblet cell hyperplasia and metaplastic substitution of highly differentiated respiratory epithelium by squamous epithelium. Epithelial changes which differ from regenerative structural alterations by manifesting cellular and nuclear atypia of varying severity are classified as potentially preneoplastic lesions of the bronchus. These can be identified as intermediate conditions between regular mucosal structures and invasive carcinomas by means of light and electron microscopy [5, 19, 21, 22]. They are almost always present in the bronchial system of patients with lung cancer.

Tumour growth and proliferation is angiogenesis-dependent. Angiogenesis is the neovascularization or formation of new blood vessels from established microcirculation [29, 30]. It is involved in a large number of normal and pathological processes in pre- and postnatal life, including wound repair, inflammation, and carcinogenesis [7, 24]. Although carcinogenesis is a complex process, tumour growth beyond 1–2 mm^3 is dependent on angiogenesis [8], which might be the result of dysregulation of the balance of angiogenic and angiostatic factors. Angiogenetic capacity, that is the property of inducing neovascularization, is characteristic of most neoplastic cells, but not of normal tissues. During angiogenesis, endothelial

cells degrade extracellular matrix while migrating towards an angiogenetic stimulus, and proliferate. Invasive growth of tumour cells implies an altered epithelial-mesenchymal interaction and in vivo investigations have demonstrated that invasion of malignant cells is always associated with marked angiogenesis. Preneoplastic and malignant cells were found to be different from benign and non-tumourigenic cells in their ability to generate a strong and directed influx of blood vessels and maintain a high degree of vascularization in the subepithelial stroma [4, 20]. We investigated whether changes in neovascularization exist in hyperplastic, metaplastic and so called preneoplastic lesions of the bronchial mucosa as potential prestages for lung cancer, using morphometric and immunohistochemical investigations.

Materials and methods

Studies were carried out on biopsy specimens from 86 patients. During routine diagnostic fibre bronchoscopy, biopsies were taken from the bronchial mucosa, mostly in cases of suspected bronchial carcinoma. Tissue specimens of bronchial mucosa including epithelium, basement membrane and tunica propria were investigated light microscopically.

Ultrastructurally, the distance between the basement membrane and the underlying fibrillar connective tissue is less than 100 µm, therefore a distinction between matrix components of the basement membrane and interstitial connective tissue is not possible in light microscopy [4]. The light microscopically visible basement membrane is a consolidation of collagen and basement membrane components and is called basement membrane zone (BM), a term introduced by Kefalides et al. in 1979 [15].

Formalin-fixed specimens, embedded in paraffin, were stained with H&E., PAS and van Gieson. Based on light microscopical criteria, specimens were divided into 9 groups: Regular bronchial mucosa (n = 12), specimens with inflammatory reaction in the tunica propria (n = 9), specimens with goblet cell- and basal cell hyperplasia (n = 24), with squamous cell metaplasia (n = 9), with squamous cell metaplasia with mild, moderate and severe dysplasia (n = 11), and 13 cases with carcinomata in situ. Additionally 9 cases with micropapillomatosis were investigated.

The grade of neovascularization was assessed by the microvessel density, which was obtained by an immunohistochemical staining of endothelial cells with factor-VIII-related antigen. A polyclonal rabbit-anti-human antibody against von Willebrand factor (Dakopatts, Code No. A 082, Lot No. 128) in a dilution 1:2000, was used in the ABC technique (Vectastain, Camon Laboratories, Wiesbaden, Germany), according to the method described by Hsu [13]. Serial paraffin sections were placed on poly-L-lysine-coated slides (10% poly-L-lysine (Sigma, Deisenhofen, Germany)), dried overnight at 37°C, deparaffinized and hydrated. Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide in TRIS buffer (pH 7.6) at 20°C for 20 min, non-specific protein binding by incubation with 10% normal goat serum before polyclonal primary antibodies at 20°C for 20 min. After washing with PBS, the sections were incubated for 30 min with a secondary biotinylated goat anti-rabbit IgG antibody, diluted to 1:200 (Vectastain-Kit, Camon Laboratories, Wiesbaden, Germany). Incubation with a complex of avidin and biotinylated peroxidase over 30 min at room temperature and colour development with 3-amino-9-ethylcarbazole (Sigma, Deisenhofen, Germany) followed, yielding a brown-coloured precipitate. Sections were counterstained with Meyer's haematoxylin, dehydrated and mounted with synthetic mounting medium (Aquatex, Merck). Omission of the primary antibodies were used as negative controls.

Morphological analyses and microvessel density were determined with an automatic image-analysing system (VIDAS, Release 2.1, Copyright by Kontron Elektronik 1991). Microvessels were counted in selected areas of highest neovascularization on a × 100 field (×10 objective lens and ×10 ocular lens; 0,6 mm^2) 0.4 mm underneath the basement membrane zone in the tunica propria. Microvessel counts and the minimal and maximal diameter of the vessels were used as morphological and morphometrical variables. In each specimen, 5 values were determined giving a mean value of microvessels identified within the ×100 field.

We used a Student's t test to analyse the results. A P value between 0.05 and 0.001 was considered to indicate a significant difference between the groups.

Results

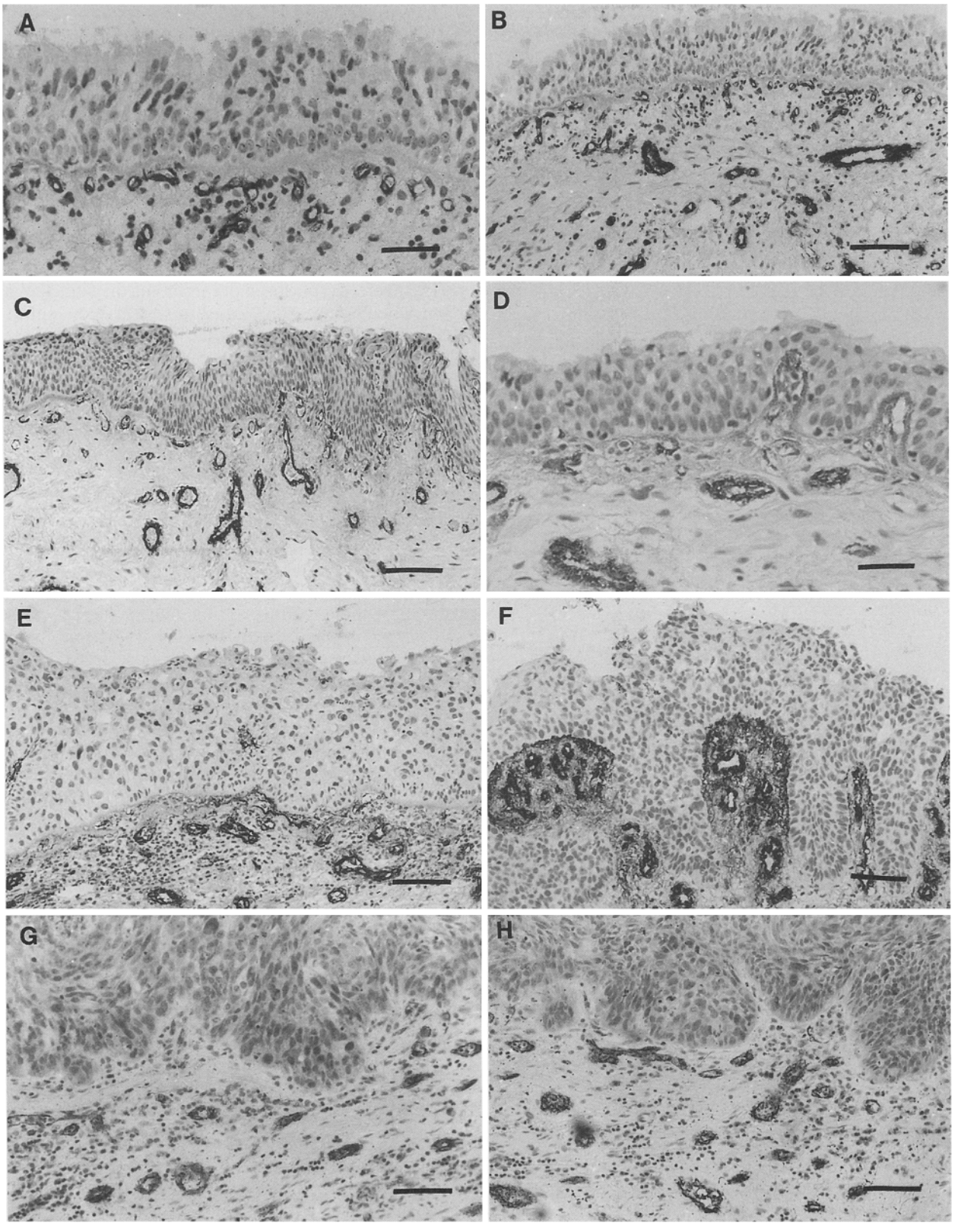
The mean microvessel count in regular bronchial mucosa was 20 vessels/0.6 mm^2 field, 0.4 mm underneath the basement membrane zone in the tunica propria with a mean vessel diameter of single vessels of 9.04×10^{-4} mm^2. In specimens with inflammation of the tunica propria with infiltration by lymphocytes and leucocytes, the mean microvessel count increased to 26 vessels/0.6 mm^2 (Fig. 1A,B) with a mean surface of cut section of 14.4×10^{-4}mm^2. In basal cell- and goblet cell hyperplasias, a mean of 24 vessels/0.6 mm^2 was measured (Fig. 1C). Mean surface of cut section was 5.38×10^{-4} mm^2.

In squamous cell metaplasia without dysplasia and inflammation of the tunica propria, a mean vessel count of 33 vessels/0.6mm^2 was found 0.4 mm underneath the basement membrane zone in the tunica propria. In squamous cell metaplasia with mild and moderate dysplasia, up to 50 vessels/0,6 mm^2 (Fig. 1E) and in squamous cell metaplasia with severe dysplasia and carcinoma in situ up to 61 vessels/0.6 mm^2 were found (Fig. 1F, G H). With increasing degree of dysplasia, increasing mean microvessel counts were found in a 0.6 mm^2 field 0.4 mm beyond the basement membrane zone in the tunica propria.

Mean surface of cut section in squamous cell metaplasia was 15.3×10^{-4} mm^2, in squamous cell metaplasia with different degrees of dysplasia mean vessel diameter (surface of cut section) was 2.95×10^{-4}mm^2 and in carcinoma in situ 9.08×10^{-4} mm^2.

Squamous cell metaplasia with severe dysplasia and carcinoma in situ were always accompanied by inflam-

Fig. 1A–H Microphotographs demonstrating factor VIII staining in regular bronchial mucosa and potentially preneoplastic lesions. **A** Bronchial mucosa with inflammatory reaction in the tunica propria (mild basal cell proliferation). Vessels can be distinguished in the tunica propria (×250, *bar* 40 µm). **B** Bronchial mucosa with inflammatory reaction in the tunica propria at an other region. Increased microvessel count in a 0.6 mm^2 field in the tunica propria (×125, *bar* 80 µm). **C** Squamous cell metaplasia with neovascularization close to the basement membrane zone (×125, *bar* 80 µm). **D** Micropapillomatosis with hernia-like protrusions of vascularized stroma papillae extending towards the epithelium (×250, *bar* 40 µm). **E**: Squamous cell metaplasia with moderate dysplasia (×125, *bar* 80 µm). **F** Micropapillomatosis and diagnosis of carcinoma in situ with increased neovascularization in stroma papillae (×125, *bar* 80 µm). **G, H** Carcinoma in situ close to a squamous cell carcinoma at other region with numerous capillary vessels in close vicinity to the disintegrated and thinned basement membrane zone (×250, factor, *bar* 40 µm)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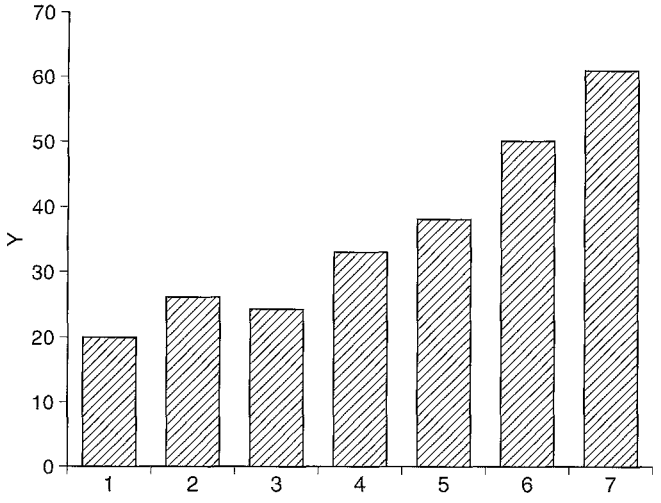

Fig. 2 Vessel count in regular bronchial mucosa, hyperplastic and potential preneoplastic lesions of the bronchial mucosa ((*Y*) vessel amount in a 0.6 mm^2 area in the tunica propria, (*1*) regular bronchial mucosa, (*2*) bronchial mucosa with inflammation, (*3*) goblet cell and basal cell hyperplasias, (*4*) squamous cell metaplasia, (*5*) micropapillomatosis, (*6*) squamous cell metaplasia with mild, moderate and severe dysplasia, (*7*) carcinoma in situ)

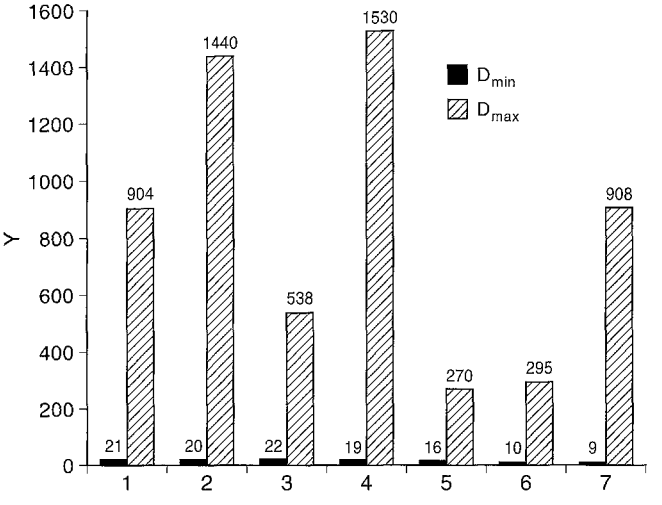

Fig. 3 Minimal and maximal vessel diameters in regular bronchial mucosa, hyperplastic and potential preneoplastic lesions of the bronchial mucosa ((*Y*) minimal and maximal vessel diameters in $\times 10^{-6}$ mm^2, (*1*) regular bronchial mucosa, (*2*) bronchial mucosa with inflammation, (*3*) goblet cell and basal cell hyperplasia, (*4*) squamous cell metaplasia, (*5*) micropapillomatosis, (*6*) squamous cell metaplasia with mild, moderate and severe dysplasias, (*7*) carcinoma in situ). Example: The minimal mean vessel diameter of regular bronchial mucosa is: D_{min}: 21×10^{-6} mm^2. The maximal mean vessel diameter of regular bronchial mucosa is: D_{max}: 904×10^{-6} mm^2

matory reactions in the tunica propria. Apart from lymphocytes, neutrophil leucocytes, macrophages and fibroblasts were found. In contrast to bronchial mucosa with inflammation in the tunica propria, the vessel density in potential preneoplastic lesions increased up to 61 vessels/0.6 mm^2, whereas surface of cut section decreased to 2.95×10^{-4} mm^2 (bronchial mucosa with inflammation in the tunica propria: 11.3×10^{-4} mm^2, (Figs. 2, 3).

Increasing neovascularization was found in correlation with an increasing degree of dysplasias, which was accompanied by an increased matrix disarrangement of the basement membrane zone. Basement membrane zone was demonstrated in H&E, PAS and van Gieson stain as a linearly intact, homogenously stained zone directly beneath the surface epithelium in specimens with regular bronchial mucosa with or without inflammatory reaction in the tunica propria, basal cell- and goblet cell hyperplasia and squamous cell metaplasia with mild and moderate dysplasia. In all specimens, microvessels were found underneath the basement membrane zone in the tunica propria.

In severe dysplasia and carcinoma in situ, the basement membrane zone was disintegrated, split and thinned out to about 2–1 μm. Numerous microvessels orientated vertically to the basement membrane zone were found in the close vicinity of the BM zone accompanied by a marked inflammatory reaction.

In all groups examined, significant differences in mean microvessel count and mean microvessel diameters were found.

Micropapillomatosis is characterized by highly vascularized portions of subepithelial stroma, mainly occurring in metaplastic areas of the bronchial mucosa. Light microscopically, hernia-like protrusions of vasculated stroma papillae extending towards the surface epithelium, with or without corresponding bulges of the epithelial surface, were found. Epithelial changes around these micropapillomatoses were similar to those found in transitional or epidermoid metaplasia. Cellular and nuclear atypia develops to a considerable degree. In cases of micropapillomatosis with metaplasia of the epithelium, a mean vessel count of 30 vessels/0.6 mm^2 was found in the stromal papillae and in an area 0.4 mm underneath the basement membrane zone in the tunica propria (Fig. 1D). In our own material cases of micropapillomatosis with a considerable degree of cellular and nuclear atypia and classified as carcinoma in situ had a mean microvessel count of up to 61 vessels/0.6 mm^2, with a predominant localization of microvessels within the stroma papillae. Numerous interepithelial sprouts of endothelial cells were seen next to protrusions of vascularized stromal papillae extending to the dysplastic epithelium (Fig. 1F).

Discussion

Angiogenesis is the formation of new blood vessels from the established microcirculation. Tumour growth and metastasis are angiogenesis-dependent events and a wide variety of non-neoplastic diseases are characterized by angiogenesis [6, 7]. Normally, physiological angiogenesis occurs infrequently, yet it can be induced rapidly in response to a number of diverse physiological stimuli. Among the most extensively studied of these angiogenesis-dependent physiological processes is normal wound repair. An important feature of wound-associated angiogenesis is that it is locally transient and tightly controlled.

The reparative processes of the bronchial mucosa due to acute and chronic injuries are expressed as basal cell hyperplasia, goblet cell hyperplasia and metaplasia. In these lesions, vascularization and neovascularization, with an increase of mean microvessel count from 20 microvessels/0.6 mm^2 in regular bronchial mucosa to 24 microvessels/0.6mm^2 in hyperplastic and 33 microvessels/0.6mm^2 in metaplastic lesions, can be interpreted as a result of wound-associated angiogenesis. Acute and chronic inflammatory reactions in the tunica propria are associated with an increase of microvessel diameter because of hyperaemia. Therefore, significant differences in single microvessel diameter (surface of cut section) were found with increase from $9.58 \times 10^{-4}mm^2$ in specimens with regular bronchial mucosa to 11.3×10^{-4} mm^2 specimens with acute inflammation and 15.3×10^{-4} mm^2 metaplastic lesions (Fig. 3).

Under conditions of normal wound repair angiogenesis appears to be under strict control, however, during neoplastic transformation of the bronchial epithelial cells it is exaggerated. With increasing degree of epithelial dysplasia and atypia up to carcinoma in situ, an increase of mean microvessel density up to 61 vessels/0.6 mm^2 was found. Severe dysplasias and carcinoma in situ are associated with marked inflammation of the tunica propria mainly by leucocytes, fibroblasts and macrophages. As monocytes/macrophages as well as leucocytes produce angiogenetic cytokines, for example vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), IL-10 or transforming growth factor (TGF) alpha, neovascularization might be induced by products of these cells [18, 26–29].However, it is clear that tumour cells themselves are able to express angiogenic cytokines, for example IL-8 [28, 29]. The finding of heterogenous expression of angiogenic factors by atypical cells and tumour cells suggest that specific subclones of neoplastic cells may exist and function as the primary cellular source of tumour-derived angiogenetic factors. The predominance of mainly small vessels with microvessel diameters of minimal $2.95 \times 10^{-4}mm^2$ points to the formation of new blood vessels from the established microcirculation in these lesions.

Micropapillomatosis with typical subepithelial vessel proliferation included all forms of metaplastic change in addition to cellular and nuclear atypias resembling those found in dysplastic areas. The interpretation of micropapillomatosis as a preneoplastic lesion is supported by the increasing incidence of such changes in the bronchial epithelium of patients with manifest bronchial carcinoma [20, 23]. Remembering the changing pattern of vascularization in the early phases of bronchial carcinoma, these vascular proliferations within the stroma papillae might be interpretated as the earliest change in the stroma of a future carcinoma.

However, when normally quiescent endothelial cells lining venules are stimulated, they will degrade their basement membrane zone and proximal extracellular matrix, migrate directionally, divide and organize into new functioning capillaries invested by a new basal lamina. As the extracellular matrix plays a crucial role in the process of endothelial cell migration, proliferation and differentiation during angiogenesis [7, 8], the conditions for the steps of tumour invasion and tumour progression are formed.

With increasing degree of dysplasia, a degradation of the BM zone with loss of extracellular matrix components like collagen type IV and laminin was found in preneoplastic lesions of the bronchus [4, 5]. A main component of the BM is basic fibroblast growth factor (bFGF), which is one of the potent angiogenetic factors. As the process of BM degradation is accompanied by neovascularization – in carcinoma in situ neovascularization was found in close vicinity of the BM zone – and BM degradation leads to a release of bFGF, so neovascularization might be induced by bFGF [29].

Although neoplastic transformation is dependent on multiple genetic and epigenetic events, the success of tumourigenesis is dependent on the complex biological interplay between the neoplastic cells and the resident and recruited host-responding cells. The impulse for endothelial cell proliferation might be given by the release of cytokines from atypical epithelial cells in potential preneoplastic lesions, in the shape of dysplasias and carcinomata in situ, or by stroma cells. Additionally, the dual growth factor phenomena of autocrine stimulation of cell proliferation and paracrine stimulation of the surrounding cells may play a role[15]. These results suggest a paracrine coordination of angiogenesis and endothelial cell proliferation which is tightly regulated and transient in embryonic tissues [29], switched off in the normal adult tissues [12] and turned on again in preneoplastic lesions of the bronchial mucosa and in lung cancer. Every tumour must continuously stimulate the growth of new capillary blood vessels for the tumour itself to grow [8]. Furthermore, the new blood vessels embedded in lung tumours provide a route for tumour cells to enter the circulation and to metastasize to distant sites, such as bone, brain and liver [31].

Our findings suggest that neovascularization takes place in the development of hyperplastic, metaplastic and potential preneoplastic lesions of the bronchial mucosa and that the "angiogenic phenotype" of preneoplastic lesions and lung tumours can be thought of as a net imbalance between regulated and unregulated processes of angiogenesis. It is important both to identify the extracellular molecules whose production (in the case of inducers of angiogenesis) or lack of production (in the case of inhibitors of angiogenesis) results in preneoplasia- or tumour neovascularization [2]. In vivo and in vitro results suggest that the temporally ordered synthesis of specific matrix components plays a significant role in orchestrating the growth and differentiation of endothelial cells during the highly integrated set of responses known as angiogenesis [10]. The varying degrees of angiogenesis in potential prestages of lung cancer or various types of lung cancer [14] may depend on the biological properties of atypical cells, such as their proliferation rate, degree of growth factor production [1] and distribution of

growth factor receptors [3, 11, 24, 25]. Previous studies on tumour growth, however, have not revealed when or how the transition to an angiogenic stage occurs during early tumour development [9]. In remembering the changing pattern of angiogenesis in the early phases of bronchial carcinoma, we also suggest that angiogenic activity and neovascularization in potential preneoplastic lesions of the bronchial mucosa are one of the earliest changes in the stroma of a future carcinoma [16].

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