Demonstration and Characterization of the Angiogenic Properties of Cervical Dysplasia

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ABSTRACT

Cervical dysplasia, or cervical intraepithelial neoplasia (CIN), is a premalignant precursor to cervical cancer. This study was designed to determine whether dysplastic lesions are angiogenic. Tissue sections from 23 surgical specimens were immunohistochemically stained for factor VIII antigen, a marker for endothelial cells. The results demonstrate that a region of neovascularization develops along the basement membrane sub-tending dysplastic epithelium when compared to adjacent normal epithelium. Comparison of microvessel counts underlying low grade lesions (condyloma and CIN I) with microvessel counts of CIN III lesions shows a statistically significant increase in the more advanced lesions. In a subset of the high grade lesions, large vascular structures are also noted in the upper layers of the epithelium, suggesting that a second stage of neovascularization consists of extension of stromal vascular papillae into the dysplastic lesions toward the surface of the epithelium. There is no statistical correlation between the amount of inflammation and the angiogenic ratio for each lesion, implying that angiogenesis is not secondary to the inflammatory response evoked by the lesion. The human papillomavirus type present in four CIN III lesions was determined by in situ hybridization; the amount of angiogenesis appears to be independent of the human papillomavirus type.

INTRODUCTION

It is now well established that invasive tumors depend on neovascularization for their continued growth, expansion, and possibly metastasis (1). Several angiogenic molecules have been identified and have been shown to be produced by tumors (1, 2). In breast and prostatic cancer, the amount of angiogenesis in the tumor is correlated with the clinical outcome, suggesting that angiogenic properties correlate with the aggressiveness of the tumor clone (3, 4). Antibodies to vascular endothelial growth factor, an angiogenic compound, inhibit the growth of human tumor cell lines injected into nude mice (5), implying that angiogenesis is required for tumor growth. Little is known about the regulation of the switch to the angiogenic phenotype. However, experiments in a transgenic mouse model system for the development of tumors of the β-cells of pancreatic islets suggest that the ability to release angiogenic factors precedes the conversion of hyperplastic islets to tumors (6). Regulation of the release of angiogenic factors may be triggered by the metabolic state of the tissue, since secretion of vascular endothelial growth factor by glioblastoma cell lines is induced experimentally by hypoxia (7).

Cervical dysplasia, also known as CIN,2 is a premalignant form of disordered growth of the stratified squamous epithelium of the cervix. The lesions represent the biological and histological precursor to invasive cervical cancer. Dysplasia is characterized histologically by the presence in the upper layers of the epithelium of mitotically active cells with enlarged, irregular nuclei and increased nuclear/cytoplasmic ratios. The lesions are graded histologically as mild (CIN I), moderate (CIN II), and severe dysplasia or carcinoma in situ (CIN III), depending upon the extent of nuclear abnormality and the degree to which the differentiated upper layers of the epithelium are replaced by abnormal cells (8). The lesions differ from cancer in that the abnormal cells are confined to the epithelium and do not transgress the basement membrane beneath it. Both cervical dysplasia and cancer are strongly correlated with the presence of HPV, particularly of the subtypes 16 and 18 (9). Clinically, dysplastic lesions are detected by Papanicolaou smear screening and localized by stereoscopic examination of the cervix in a technique known as colposcopy. As viewed by colposcopy, lesions appear as white plaques after the application of acetic acid and can be graded by the presence of red patterns of punctuation or mosaicism on the white background, which represent vascular structures within the dysplasia (10). On occasion, atypical vessels can be seen colposcopically on the surface of the lesion and are associated with frank invasion on biopsy (11). The presence of macroscopic vascular patterns with dysplastic lesions would suggest that an angiogenic event is associated with dysplasia. This study was designed to test this assumption. As a premalignant lesion with distinct histological grades preceding the development of cancer, this entity affords an opportunity to dissect the natural history of the development of the angiogenic phenotype in a naturally occurring neoplasia.

MATERIALS AND METHODS

Patients who had undergone cervical cone biopsies or loop excisions were identified from the files of the Dysplasia Clinic at the University of California at San Francisco. Sections of the surgical specimens were cut from the formalin-fixed, paraffin-embedded tissues obtained from the pathology archives. Where possible, blocks were chosen that contained both normal and abnormal histology on the same section. Sections were deparaffinized, digested with trypsin and Pronase, and incubated with a 1:6000 dilution of a polyclonal antiserum to factor VIII-related antigen (DAKO Corp.). Immunoperoxidase staining was then performed by incubation with a peroxidase-conjugated secondary antibody and reaction with diaminobenzidine and hydrogen peroxide. Sections were counterstained with hematoxylin. Specimens were examined on an Axioscope microscope with bright field illumination with the ×10 ocular and the entire lesion was photographed in overlapping fields using Kodak Ektar film ASA 100. The prints were pasted together to make a montage of the entire lesion and the adjacent normal tissue. The number of factor VIII-staining structures underlying normal cervical epithelium and dysplasia was then counted on the photographs and the lengths of the basement membranes were measured. The values were expressed as vessels/cm of normal or dysplastic epithelium. The angiogenic ratio for each specimen is the number of vessels/cm under the lesion divided by the number of vessels/cm under normal epithelium.

To assess the degree of inflammation present in the epithelium, adjacent sections were also immunostained with a monoclonal antibody to a macrophage marker, CD68 (KP-1, 1:1000 dilution; DAKO Corp.). The number of CD68-positive cells/unit area was counted on photographs of normal epithelium and adjacent lesions; the inflammation ratio is the number of stained cells/unit area of dysplastic tissue divided by the number of stained cells/unit area of normal tissue. HPV typing of tissue sections was performed using a Digene Tissue Hybridization Kit, following the manufacturer’s instructions. Statistical analysis was performed by a two-tailed Mann–Whitney U test or Spearman’s rank correlation using True Epistat statistical software. P = 0.05 (two-tailed) was considered significant.

Received 11/8/93; accepted 11/24/93.

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1Recipient of a Reproductive Scientist Development Program award through the National Institute of Child Health and Human Development and the American Gynecological and Obstetrical Society.

2The abbreviations used are: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.
RESULTS

Factor VIII Staining of Normal and Dysplastic Epithelium. Twenty-three lesions were immunostained with antibodies to factor VIII antigen. The specimens represented 3 condyloma, 3 CIN I, 6 CIN II, and 11 CIN III lesions. CIN lesions demonstrate enhanced factor VIII staining compared with normal adjacent epithelium (Figs. 1 and 2). The effect is confined to the region immediately beneath the epithelium and does not extend into the stroma below. High power microscopy revealed an extensive reticular network of vessels underlying the lesions compared with normal epithelium (Fig. 1, C and D). In addition, the areas of neovascularization are sharply demarcated between the dysplastic and normal tissue (Fig. 1, E and F).

Endocervical stroma contains mucus-secreting endocervical glands which are lined by columnar epithelium. These glands are not angiogenic as detected by factor VIII staining (Fig. 2A). Columnar epithelium can be replaced by squamous epithelium through the normal process of metaplasia; when dysplasia is present, dysplastic cells can replace the glandular epithelium. Fig. 2B shows such a gland in the process of being replaced by dysplasia. There is pronounced factor

Fig. 1. Enhanced staining for factor VIII antigen immediately beneath the basement membrane subtending dysplastic epithelium. Representative fields of normal (A and C) and dysplastic (B and D) lesions were photographed through an Axioshot microscope. Lesions were histologically graded as CIN III (B and F) and CIN II (D and E). N, normal epithelium; D, dysplasia. Bar: 100 μm for A, B, E and F; 50 μm for C and D.

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VIII staining surrounding that part of the gland involved in the dysplastic process, and again the angiogenic response is confined to the regions immediately subtending the dysplastic epithelium. This phenomenon was observed in many examples of endocervical glands containing dysplasia.

Comparison of the vessel counts of condyloma and CIN I lesions with the counts of adjacent tissue showed no significant difference \( (P = 0.31 \text{ by Mann-Whitney } U \text{ test}) \). Comparison of the vessel count from CIN II and CIN III lesions with that from the adjacent normal tissue showed a statistical difference \( (P = 0.0006) \). Since there was a wide range in the values for both the normal tissue and the lesions, the data were also expressed as a ratio of the vessel count of the lesion divided by the normal epithelium from the same patient (angiogenic ratio). Comparison of the angiogenic ratios with the grade of CIN revealed that the degree of angiogenesis increases with the severity of the lesion (Fig. 3). The difference between the angiogenic ratios of the less advanced lesions (condyloma and CIN I) and the CIN III lesions is statistically significant \( (P = 0.014 \text{ by Mann-Whitney } U \text{ test}) \), whereas the difference between the angiogenic ratios of the CIN II lesions and the CIN III lesions shows no statistically significant difference \( (P = 0.08) \). These data suggest that the angiogenic properties of CIN become more pronounced as the lesion progresses toward more advanced histology.

**Presence of Factor VIII-staining Structures within the Epithelium.** In addition to the enhanced staining for factor VIII-related antigen in the boundary between the epithelium and the stroma, some specimens revealed the presence of numerous, large factor VIII-positive structures within the epithelium itself (see Fig. 1, B and E). These structures presumably reflect stromal vascular papillae extending into the upper levels of the dysplastic epithelium. These vascular structures were present in 5 of 11 (45%) CIN III lesions, 1 of 6 (16%) CIN II lesions, none of the CIN I/condyloma lesions and none of 23 samples of normal epithelium. Statistical analysis confirms that the presence of stromal vascular papillae within the upper half of the epithelium is correlated with the grade of the dysplasia \( (\phi^2 = 14.6, P = 0.002) \). Comparison of the angiogenic ratios of the CIN III lesions containing vessels in the upper half of the epithelium with those not containing them revealed no statistical difference \( (P = 0.073 \text{ by Mann-Whitney } U \text{ test}) \). This result suggests that growth of the stromal vascular papillae into the upper layers of the epithelium does not represent a continuation of the process of neovascularization along the basement membrane but rather a separate process.

**Measurement of Inflammation in Dysplastic Epithelium.** Dysplastic tissues elicit a host immune response, and inflammatory cells are known to contain and secrete many angiogenic compounds (12). Accordingly, the appearance of angiogenesis in dysplastic tissues may reflect the host response to the lesions and may not be induced by the dysplastic cells themselves. To address this possibility, tissue sections from 18 lesions were stained with antibodies to CD68, a macrophage marker. The inflammatory ratio (CD68-positive cells/unit area in dysplastic tissue divided by CD68-positive cells/unit area in normal tissue) was compared with the angiogenic ratio for each lesion (Fig. 4). There was no statistical correlation between the angiogenic and inflammatory ratios. These data suggest that the angiogenic phenotype is independent of the amount of inflammation present and therefore may reflect a property of the dysplastic cells themselves.

**HPV Typing of CIN III Lesions.** The development of cervical dysplasia and cancer is strongly correlated with the presence of HPV. Certain strains of HPV, most commonly HPV 6 and 11, are associated with condyloma and only rarely with CIN or cancer, whereas HPV 16 and 18 are most commonly associated with cancer; an intermediate risk group (HPV 31, 33, 35, 51, 52, and 58) is associated with CIN and less commonly with cancer (13). We noted a marked heterogeneity in
the angiogenic ratio of the CIN III lesions, with some lesions having almost no neovascularization (angiogenic ratio, 1.2) and others appearing markedly angiogenic (angiogenic ratio, 4.4). It is possible that HPV types associated with a more aggressive histology might invoke a stronger angiogenic response; we therefore tested whether the degree of angiogenesis is a reflection of the HPV type present in the lesion. HPV typing was performed on a subset of CIN III lesions representing 2 lesions with a low angiogenic ratio (1.2) and 2 lesions with high angiogenic ratios (2.3 and 4.4). Of the lesions with an angiogenic ratio of 1.2, one was found by in situ hybridization to have HPV 16/18 and the other had HPV 6/11. Both of the lesions with the high angiogenic ratios had positive staining for HPV 30s. Thus, the HPV type with the highest malignant potential was found in a lesion with the lowest angiogenic ratio, and the lesions with the highest angiogenic ratios contained the HPV types with the intermediate malignant potential. Therefore, on this limited subpopulation of lesions, no obvious correlation was found between the angiogenic phenotype and the malignant potential of the HPV type.

**DISCUSSION**

Dysplastic lesions show enhanced staining for factor VIII antigen compared with adjacent normal cervical epithelium in a process which is variable but which is significantly enhanced in the CIN III lesions. The neovascularization is confined to a narrow zone immediately underlying the dysplastic epithelium and extends neither laterally nor inferiorly from this zone. Endocervical glands replaced by dysplastic cells also demonstrate angiogenesis, indicating that the effect is not dependent on the location of the epithelium on the surface of the cervix. A subset of CIN II and III lesions demonstrate a second type of neovascularization, consisting of the presence of vascular stalks in the upper layers of the epithelium where they are not normally found. The fact that neovascularization is most pronounced in the advanced lesions suggests either that enhanced vascular supply reflects the increased metabolic activity of the lesion or that angiogenesis is an important prerequisite for the subsequent development of invasive clones. It is not possible to correlate the natural history of the lesions with the angiogenic ratios since these lesions were excised for treatment.

Previous studies have documented that preneoplastic lesions possess angiogenic properties. For example, tissue from mouse and human mammary hyperplasia elicits an angiogenic response when transplanted onto the rabbit iris, whereas normal breast epithelium does not (14, 15). Human breast carcinoma in situ shows neovascularization as detected by factor VIII staining (3). Preneoplastic bladder epithelium, but not normal bladder epithelium, elicits an angiogenic response when implanted in the rabbit iris (16). In a transgenic mouse model system for the development of fibrosarcomas, the angiogenic compound basic fibroblast growth factor is made by premalignant and malignant lesions but is secreted only by those lesions which have progressed to the tumorigenic stage (17). In a transgenic model system for the development of tumors of the β-cells of the pancreatic islets, angiogenesis appears in a subset of hyperplastic cells before they become neoplastic at a frequency which correlates with tumor development (6). These studies suggest that angiogenesis may precede the development of invasive tumors in certain cases.

Our data constitute the first demonstration of the natural history of the switch to an angiogenic phenotype in a naturally occurring human preinvasive lesion and suggest that the neovascularization that accompanies cervical dysplasia occurs in a series of stages. The earliest event appears to be the elaboration of a localized complex microvasculature subtending the abnormal growth, suggesting the diffusion of angiogenic compounds from the dysplastic cells. Since the degree of angiogenesis does not correlate with the amount of inflammation in the lesion, the angiogenic response likely reflects signals generated by the dysplastic cells themselves. In a subset of advanced lesions, vascular stalks are also present within the upper thickness of the epithelium, suggesting that the next stage in angiogenesis involves the elaboration of the factors that allow the extension of stromal vascular papillae into the epithelium. It is not known whether these structures would be detected colposcopically as punctuation and mosaicism. It is also not known whether the vascular stalks derive from preexisting stromal vascular papillae or reflect further elaboration and maturation of the reticular network of microvessels lining the basement membrane. Hypothetically, in the next stage of neovascularization, the vessels would reach the surface of the epithelium and would be detected colposcopically as atypical vessels, at which point the lesion would have progressed to cancer (11).

Thus dysplasia may serve as a useful model for the dissection of the stages involved in the neovascularization of a tumor. Whether the molecular basis of these phases represents the continuum of a single process or discrete processes is at present unknown and requires elucidation of the factors responsible for the angiogenic response described here. It is interesting to speculate that the angiogenesis might be necessary for the growth, maintenance, or progression of dysplastic lesions. If so, antiangiogenic compounds might have a therapeutic role in the clinical management of dysplasia.

**ACKNOWLEDGMENTS**

We thank J. M. Bishop for helpful suggestions throughout the project and for financial support; J. M. Bishop and R. Weiner for critical reading of the manuscript; and L. Deiss, R. Weiner, and Z. Werb for useful discussions.

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