Ultrastructural and Morphometric Study of Diethylstilbestrol-associated Lesions Diagnosed as Cervical Intraepithelial Neoplasia III

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ABSTRACT

Investigations were carried out to characterize diethylstilbestrol (DES)-associated squamous lesions and to assess their biological significance. Five DES-associated cervical lesions displayed architectural features which were diagnosed as cervical intraepithelial neoplasia (CIN) III, such as full-thickness replacement by atypical squamous cells with vertical orientation and absence of normal polarity. Electron microscopic examination revealed only one of the five to be consistent with the generally recognized ultrastructural picture of non-DES CIN III. In the remaining four lesions, the moderate-to-large numbers of tonofilibrils and well-developed desmosomes distinguished them from the true CIN III lesions. Morphometric studies indicate the five DES-associated lesions in this study as a group to be significantly different from normal squamous epithelium, from maturing metaplasia, and from non-DES-related CIN III in the parameters of differentiation studied. Their intermediary position between maturing metaplasia and non-DES CIN III suggests that they are more differentiated than CIN III and less differentiated than maturing metaplasia. Nuclear area measurements indicate the increased nuclear-cytoplasmic ratio observed in the DES-associated CIN III lesions of this study is due to a decrease in cytoplasmic volume, as opposed to an increased nuclear size.

INTRODUCTION

The gross and microscopic features of vaginal adenosis found in young females exposed prenatally to DES have been well documented (1, 3, 4, 8, 9, 12, 16–20). Metaplastic squamous epithelium forms the variable transformation zone resulting from the so-called "healing phase" of vaginal adenosis. Considerable controversy has been generated concerning the biological potential of squamous cell lesions of the cervix and vagina arising in such transformation zones commonly diagnosed as various grades of CIN (2, 6, 7, 11, 13, 15). The consequent formation of extensive transformation zones in areas of vaginal adenosis has prompted some to envision squamous cell neoplasms rather than glandular ones as the predominant source of future problems for these young women (2, 11). These workers have postulated that the large transformation zones provide more surface area of young active epithelium for exposure to potential carcinogens. Furthermore, many of these patients have not yet reached the age at which CIN becomes a problem. The combination of these factors, plus the current trend toward earlier and more frequent sexual encounters, has led some to speculate that these patients will have a higher incidence of squamous neoplasia than will those not exposed to DES. Although this theory appears reasonable, other investigators have questioned its validity, especially in light of recent reports regarding CIN-like squamous lesions in DES-exposed young women. Among 1424 young women exposed in utero to DES, Robboy et al. (14) found both prevalence and incidence of squamous dysplasia to be low; when present, dysplastic changes were usually mild in those women without prior history of atypia. CIN III (severe dysplasia, carcinoma in situ) was observed only in those patients with the referring diagnosis of such lesions. These investigators believed that it was most important to make the distinction between dysplasia and metaplasia, considering the widespread occurrence of metaplasia in the transformation zones of DES-exposed patients. If this histological distinction was not appreciated, a disproportionate high percentage of dysplastic lesions could potentially be diagnosed in these patients.

Various workers have reported dissimilarity in prevalence rates of CIN lesions in DES-exposed progeny. Mattingly and Stafl (11) reported the prevalence rate of carcinoma in situ to be nearly 5 times that of the DES-unexposed control group. In a double-blind placebo controlled cytological study, Bibbo et al. (2) found in progeny exposed in utero to DES a significantly increased rate of mild to moderate dysplasia. In contrast, Richart et al. (13) in a microspectrophotometric study of squamous lesions associated with prenatal DES exposure, found no increased rate of cervical or vaginal intraepithelial neoplasia. In fact, of 26 cervical and vaginal lesions, all of which had been diagnosed by referring physicians as having some degree of dysplasia or carcinoma in situ, 10 were subsequently judged to be mature or maturing physiological metaplasia, 7 were considered atypical metaplasia, and only 9 were called true intraepithelial neoplasia. They believed that these findings support the concept that squamous neoplasms associated with prenatal DES exposure are frequently overdiagnosed. Furthermore, when these lesions were studied with DNA microspectrophotometry, all lesions representing physiological squamous metaplasia were found to have a diploid DNA content, whereas virtually all the atypical metaplasias had bimodal peaks of diploid-tetraploid chromosome number. Squamous lesions judged to be CIN III were discovered to have variable ploidy with tetraploid, hypertetraploid, and aneuploid values. Of the 9 lesions, 6 had either tetraploid or hypertetraploid DNA content and 3 had aneuploid DNA content.

The histological picture most easily confused with squamous dysplasia in DES-exposed progeny is maturing squamous metaplasia. This is due to the presence in the metaplastic epithelium of fewer histological characteristics indicative of
squamous differentiation, such as prominent intercellular bridges, glycogenation in upper levels of epithelium, and initiation of stratification in upper levels. In addition, cell borders are less well defined than in mature metaplasia or original epithelium, and nuclei are more monotonous throughout the different strata of epithelium.

Due to the controversy engendered over the significance and biological potential of cervical and vaginal CIN III-like lesions in DES-exposed young women, an effort was made to procure suitable specimens for study. This report entails an ultrastructural and morphometric study of 5 such lesions diagnosed as CIN III in women exposed to DES in utero. These lesions are morphometrically compared to an equal number of examples of normal squamous epithelium, maturing metaplasia, and non-DES-related CIN III. Morphometric studies were undertaken to attempt some degree of quantitation of parameters used subjectively in evaluation of the squamous neoplastic lesions.

MATERIALS AND METHODS

Material for study was obtained by colposcopically directed biopsy of the cervix from 5 young females who had been exposed to DES in utero and whose ages ranged from 20 to 25 years. Patients were initially identified either by abnormal Papanicolaou smears or a positive history of prenatal exposure to DES. Following colposcopic examination in which the transformation zone was identified and its extent determined, any abnormal areas were biopsied. Similarly, material was obtained from 5 non-DES-exposed CIN III cervical lesions, from 5 cervixes undergoing physiological squamous metaplasia, and from 5 normal cervices covered by mature squamous epithelium. Tissue was processed by fixation in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Biopsies submitted for ultrastructural study were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.6 M sodium cacodylate buffer (pH 7.4) for 3 hr, washed, and postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 2 hr. The tissue was then dehydrated using graded acetone solutions, and infiltrated and embedded in Durcupan ACM resin. After polymerization, the tissue was sectioned on an MT II ultramicrotome using a diamond knife. The sections were stained with a saturated solution of uranyl acetate and Reynolds' lead citrate, then examined in a Zeiss EM-9S-2 electron microscope.

Morphometric studies of each of these 4 groups were accomplished in the following manner. Transmission electron micrographs of the middle third of the epithelium from the 4 patient groups were printed at a standard magnification of X4500. Cytoplasmic and nuclear outlines were traced with a line-graph tracer pen interfaced to a Hewlett-Packard calculator. NCR and NA (sq μm) were computed from these measurements. Desmosomes per unit length of cell membrane were point counted, utilizing the same tracer pen.

RESULTS

Light and Transmission Electron Microscopic Studies. All 5 DES CIN III lesions had the following histological features, although to varying degrees: absence of nuclear polarity in relationship to the basement membrane with vertical orientation of cells; some degree of nuclear hyperchromatism; and crowding of cells with poor delineation of cell borders. Mitoses varied from specimen to specimen but were generally increased in number; abnormal mitoses were identified in all lesions. Light and transmission electron microscopy revealed abnormal mitoses to be in the form of tripolar mitoses, abnormalities of the spindle apparatus, 2 group metaphases, ring forms, and other bizarre patterns (Fig. 1). Intracellular glycogen was notably absent from all lesions.

Case 1 was a cervical lesion in which the epithelium was arranged in a scalloped pattern (Fig. 1a). Deep and narrow indentations of the stroma containing a prominent and dilated capillary vasculature were interposed between rete pegs. The epithelium was occupied to varying levels by deeply eosinophilic, vertically oriented cells with pleomorphic nuclei. Although some nuclei were hyperchromatic, others had a finer speckled nuclear pattern, and still others demonstrated a loose pale chromatin distribution and appearance. Mitoses averaged 1 to 2/ high-power field. Cell borders were frequently distinct, and fairy prominent intercellular bridges were present. Demarcation between this eosinophilic layer and the overlying cells, when present, was marked. Cells in the more superficial layers were large and palely eosinophilic with crowding and continued absence of polarity. Only a very thin rim of slightly flattened cells remained on the surface. Perinuclear glycogen vacuoles were frequently present.

Ultrastructural examination of representative areas disclosed cells of high NCR containing large nuclei with small, usually single, nucleoli (Fig. 2). Chromatin was evenly dispersed in a finely granular pattern with sparse heterochromatin. Nuclear outline was regular with bizarre indentations. Squamous elements such as tonofilbrils and desmosomes were present; tonofilbrils were numerous and desmosomes were moderately well formed.

Case 2 was a cervical lesion arising in the transformation zone. The epithelium was likewise thrown into a broad scalloped pattern with narrow deep extensions of stroma between the rete pegs (Fig. 1b). Cells composing the epithelium were a monotonous population with oval to round, vertically oriented nuclei interspersed with spindle-shaped hyperchromatic cells. Many nuclei had a finely reticulate pattern. Cell borders were indistinct in some areas but readily discernible in others, with fairly prominent intercellular bridges. Mitoses were rare.

Electron microscopic observation disclosed cells with high NCR and nuclei with characteristically deep and bizarre indentations (Fig. 3). Chromatin was generally finely dispersed, and nucleoli were small and usually single. Desmosomes were few and poorly formed, and tonofilbrils were moderately numerous. Cell borders were thrown into numerous microvillous projections.

Case 3 represented a cervical lesion with epithelium composed primarily of cells lacking polarity, some of which possessed elongated spindle-type nuclei, and others of which contained more oval to round nuclei with finely speckled chromatin (Fig. 1c). Nucleoli were large and mitoses were rare. Cell borders were fairly distinct and intercellular bridges were visible.

Ultrastructural study confirmed the squamous nature of these cells in that desmosomes were abundant and well formed; concomitantly, tonofilbrils were profuse and scattered in a perinuclear distribution, as well as throughout the cytoplasm (Fig. 4).
Case 4 was a cervical lesion in which variable levels of epithelium were replaced by eosinophilic vertically oriented cells with prominent oval to spindle-shaped nuclei and poorly defined cell borders (Fig. 1d). Focally, these cells reached the surface without stratification, resulting in full-thickness replacement of the epithelium. Mitoses averaged 1 to 2/high-power field. In those areas not occupying full thickness of the epithelium, the overlying cells were atypical and more palely eosinophilic with round to oval nuclei and disordered maturation. A thin layer of flattened cells parallel to the basement membrane was observed at the surface.

Electron microscopic examination revealed cells with squamous elements, namely, moderate numbers of fairly well-formed desmosomes and large numbers of tonofilbrils (Fig. 5). Microvilli were numerous. Nuclei were large with bizarre indentations and chromatin varied from fine to coarse. Furthermore, occasional clumping of chromatin was observed.

Case 5 was a cervical lesion composed of virtually full thickness of vertically oriented cells with poor cytological delineation of cell borders (Fig. 1e). Cells exhibited absence of polarity, nuclear pleomorphism, hyperchromatism, and irregular, cleared areas in the chromatin. Nuclei were often lobed and displayed irregular borders.

By transmission electron microscopy, histological observations were confirmed with cells containing large nuclei, often with bizarre deep invaginations (Fig. 6). Nucleoli were often large and multiple, and chromatin was coarsely clumped in an irregular pattern throughout the nucleoplasm. Microvilli at the cell surface were very numerous with few poorly formed desmosomes interconnecting cells. Other indicators of squamous differentiation such as tonofilbrils were very few in number. Tripolar mitoses were identified at both the light and electron microscopic levels (Fig. 7, a and b).

In mature cervical squamous epithelium, the NCR is an important indicator of differentiation; with increasing differentiation from the basal cell layer to the flattened squamous cells of the surface layer, there is a progressive diminution in NCR. Varying amounts of glycogen dispersed or in pools is a common finding in the upper layers of the cervical epithelium, and the presence of glycogen in squamous cells is a good marker of differentiation.

The middle third of normal cervical squamous epithelium (Fig. 8) is conspicuous for several ultrastructural characteristics. Glycogen deposits are most prominent, often to the extent of occupying one-third to one-half of the cytoplasm. The cytoplasm of these cells, the long axis of which is parallel to the epithelial surface, is almost devoid of mitochondria. The nuclei are round to oval, and chromatin is finely dispersed. Desmosomes are well formed and numerous.

A corresponding epithelial layer of a physiologically maturing metaplasia is shown in Fig. 9. Conspicuous cytoplasmic features include numerous perinuclear mitochondria, abundant polyribosomes, and short bundles of tonofilbrils. These are features of an immature but metabolically active epithelial cell. Desmosomes are well formed but not as numerous as in mature squamous epithelium. Furthermore, in contrast to the middle third of mature differentiated squamous epithelium, there is an absence of glycogen pools.

Comparing the ultrastructure of maturing metaplasia and of mature squamous epithelium to non-DES-associated CIN III, it is noted that CIN III lesions are composed of poorly differ-
Those lesions were diagnosed as CIN III by surgical pathologists, as well as by the referee gynecological pathologist (H. Gore). The issue which is currently under debate and the subject of much investigation is whether these lesions are true CIN III or forms of immature or atypical squamous metaplasia mimicking CIN III. Collectively, all 5 DES lesions display full-thickness replacement by cytoplastically atypical cells which are, in general, vertically oriented, demonstrate absence of normal polarity and stratification, and are devoid of cytoplasmic glycogen. In addition, cell borders are indistinct and intercellular connections are not prominent. In other areas within the lesions, abnormal mitoses were a more prominent feature. In light of the recent work by Fu et al. (6) correlating ploidy pattern and histology of these lesions, the most reliable criterion of aneuploidy was the presence of abnormal mitotic figures; no abnormal mitoses were observed in polyploid lesions. If one extrapolates the findings of Fu et al. to this study, all 5 cases had areas with abnormal mitoses and, accordingly, aneuploid distribution; however, other areas in each lesion were not characterized by the presence of abnormal mitoses and by microspectrophotometry might be determined to have polyploid patterns. Lesions were studied with the electron microscope to afford a more discerning characterization of subcellular organelles. In this way, ultrastructural findings could be compared to available electron microscopic data concerning CIN, as well as physiological metaplasia (10, 21, 23). The current ultrastructural studies disclosed all 5 CIN III DES lesions to possess features indicative of metabolically active cells; free ribosomes, often aggregated in the form of polyribosomes, are particularly numerous, and mitochondria are likewise abundant. The absence of intracellular glycogen in all these lesions, especially in upper levels, is characteristic of immature metaplasia as well as carcinoma in situ. In both of these entities, the lack of glycogen is a reflection of failure to differentiate to more mature cells. Cell surfaces are thrown into numerous microvillous projections which interdigitate with those of neighboring cells. Numbers of desmosomes and their degree of formation are decreased as compared to those found in normal cervical squamous epithelium and maturing metaplasia. Case 5 of the DES group, however, displays the most numerous microvilli as well as the least numerous and least well-formed desmosomes of all the CIN III DES lesions. From our previous ultrastructural studies of CIN, a striking increase in cell surface projections is a constant finding in these lesions. Likewise, concomitant decrease in desmosomes which parallels severity of the lesion is another hallmark of CIN. Although the number and differentiation of desmosomes is decreased in all the DES lesions, Case 5 exhibits a striking diminution in both parameters. In fact, except for Case 2, which had the least well-developed desmosomes of the remaining 4 cases, the majority of cells in these lesions had moderately well-formed desmosomes. In addition, Case 5 had few to absent tonofibrils, whereas the other 4 lesions possessed moderate to large numbers of these cytoplasmic structures. When considering, therefore, the most reliable cytoplasmic indicators of squamous differentiation, Case 5 is most consistent with the ultrastructural picture of true CIN III. In our experience, the finding of moderate to large numbers of cytoplasmic tonofibrils and large numbers and better differentiation of desmosomes suggests that while the light microscopic picture is that of CIN III, the electron microscopic picture in Cases 1 to 4 is dissimilar to CIN III.

The most consistent nuclear ultrastructural characteristic
CIN III is coarse and irregular clumping of nuclear chromatin. This feature was found only in Case 5; nuclei of other lesions varied from evenly dispersed finely granular chromatin patterns to increased but evenly distributed heterochromatin patterns. In addition, nucleoli in Case 5 were particularly large and often multiple, whereas in the remaining 4 DES cases these structures were variably sized but usually single.

From an overall ultrastructural viewpoint, therefore, Case 5 is consistent in all respects with a CIN III lesion. The remaining 4 represent intraepithelial lesions of an ultrastructural pattern different than that of CIN III, although exhibiting full-thickness replacement of the epithelium with cytologically atypical cells by light microscopy.

Morphometric studies were undertaken to analyze more objectively the various parameters utilized subjectively in the diagnosis of squamous neoplasms. Measurements of NCR, NA and numbers of desmosomes per unit length of cell membrane were used to achieve this end. Normal middle third squamous cells showed the lowest mean NCR, as would be expected from differentiated or differentiating cells. Likewise, cells from CIN III had the highest mean NCR, as also might be expected from relatively primitive basal-type neoplastic cells. Maturing metaplasia had cells with a somewhat higher absolute mean NCR; however, there was no significant difference in NCR at the 5% level between normal middle third of the squamous epithelium and maturing metaplasia. This appears to support the physiological nature of maturing metaplastic epithelium and its close relation to native squamous epithelium of the cervix.

Mean NCR for the CIN III DES lesions was significantly higher than either normal squamous epithelium or maturing metaplasia (p < 0.01). NCR for these lesions, however, was also significantly lower than that observed for CIN III (p < 0.01). The significance of the intermediary position of the DES-associated CIN III lesions between physiological squamous epithelium and neoplastic epithelium will be discussed subsequently.

Measurement of NA among the 4 groups of patients discloses an interesting observation. Although mean NA appeared to be somewhat lower in the CIN III DES lesions, statistical analysis revealed no significant difference in nuclear area among the 4 patient groups. This is interesting information in that nuclear size measured in the middle third of the epithelium appears to be relatively constant over a range of cellular entities from normal physiological squamous epithelium, including maturing metaplasia, to the lesions of CIN III measured in this level of epithelium. This is in contrast to the work reported by Foraker (5), who observed the nuclei of carcinoma in situ to be significantly larger than those of normal cervical squamous epithelium or squamous metaplasia. It would appear that the increased NCR observed in cervical intraepithelial lesions of this study is due primarily to a decreased complement of cytoplasm, with nuclear size remaining constant.

Regarding measurements of NCR and numbers of desmosomes, morphometric studies reveal the CIN III DES lesions studied in this series to occupy an intermediary position between lesions of CIN III (not DES) and maturing metaplasia. This may indicate the CIN III DES group, when comparing these 2 parameters, to be more differentiated than other CIN III lesions but less differentiated than maturing metaplasia.

In summary, 5 cervical epithelial lesions diagnosed as CIN III in DES-exposed patients were studied by light microscopy, transmission electron microscopy, and morphometric methods in an effort to characterize these lesions which have engendered considerable controversy in the literature. Only one of these 5 CIN III DES lesions possesses fine structural characteristics of non-DES CIN III; the remainder exhibit a different electron microscopic picture with nuclear and cytoplasmic features more compatible with lesser degrees of CIN or some form of metaplasia. Morphometric studies of NCR and numbers of desmosomes of middle third epithelium indicate the CIN III DES group to be significantly different from native cervical squamous epithelium, maturing metaplasia, and non-DES-related CIN III. The findings in this study raise a question of the biological potential of the DES-associated squamous lesions and their place in the CIN spectrum.

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REFERENCES


Fig. 1. Histological sections of Cases 1 to 5 (1a to 1e) of the DES-associated CIN III lesions. Although they vary somewhat in appearance, they were determined to be CIN III by at least 2 pathologists. × 650.
Fig. 2 to 6. Electron micrographs of the DES-associated CIN III lesions (middle third of epithelium).

Fig. 2. Case 1. Cells contain large bland nuclei with generally smooth nuclear outline and sparse heterochromatin. Microvilli are numerous, but desmosomes are few and only moderately well formed. Polyribosomes are abundant and scattered diffusely throughout the cytoplasm. Glycogen is notably absent. × 8,000.

Fig. 3. Case 2. Bizarre indentations of nucleus are striking, and nucleoli are small. Finger-like microvilli interdigitate with neighboring cells, and desmosomes are few and poorly formed. Abundant mitochondria and polyribosomes are present. × 8,000.
Fig. 4. Case 3. Note vertically oriented elongated cells with prominent nuclei and variable chromatin pattern. Tonofilbrils are scattered throughout the cytoplasm; mitochondria and polyribosomes are frequent. Profiles of granular endoplasmic reticulum may be observed. × 8,000.

Fig. 5. Case 4. Cells contain nuclei with coarser chromatin pattern as evidenced by increase in heterochromatin. Microvilli are increased in number with moderately well-formed and abundant desmosomes. Cytoplasm displays coarse bundles of scattered tonofilbrils and numerous polyribosomes. × 8,000.
Fig. 6. Case 5. Cells possess irregularly shaped nuclei with coarse clumped heterochromatin pattern. Nucleoli are large and often multiple. Microvilli are numerous but short with few poorly formed desmosomes. × 8,000.

Fig. 7. a. Case 1. Light micrograph of tripolar mitosis in lower level of epithelium. × 1,600. b. electron micrograph of tripolar mitosis confirming abnormal chromatin configuration. Note absence of normal metaphase plate and clumping of chromatin into 3 interconnecting wings (arrows). × 12,000.
Fig. 8. Normal intermediate cell of cervix. Note cells with low NCR and bland nuclear chromatin pattern. Microvilli are numerous and desmosomes are moderately to well formed. Pools of glycogen form the most conspicuous cytoplasmic feature. Mitochondria and polyribosomes are notably sparse. × 8,000.

Fig. 9. Maturing metaplasia of cervix. The nuclei of these cells have finely dispersed chromatin and prominent nucleoli. Polyribosomes and mitochondria are abundant, as are short profiles of granular endoplasmic reticulum. Cell surfaces interdigitate through numerous microvilli with moderately to well formed desmosomes. Glycogen is sparse, and tonofilbrils are scattered about in short bundles. × 8,000.