Title

Temporal and spatial factors in diethylstilbestrol-induced squamous metaplasia in the developing human prostate

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Publication Date

1995

Peer reviewed|Thesis/dissertation

TEMPORAL AND SPATIAL FACTORS IN DIETHYLSTILBESTROL-INDUCED SQUAMOUS METAPLASIA IN THE DEVELOPING HUMAN PROSTATE: PERSISTENT CHANGES AFTER REMOVAL OF DIETHYLSTILBESTROL

by
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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

ORAL BIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

ABSTRACT

To determine if the metaplastic effects of diethylstilbestrol (DES) on prostatic development are reversible, human fetal prostates (obtained from abortus specimens 6-22-weeks-old) were bisected mid-sagittally; one half was grafted under the renal capsule of untreated, athymic, male nude mice and the contralateral half was similarly grafted into DES-treated hosts. Severe squamous metaplasia seen in the prostatic ducts after one month of continuous DES exposure either disappeared entirely or became reduced in extent and degree after retransplantation of the DES-treated specimens to untreated, intact male hosts and 2 additional months of growth. However, 14 of 21 DES-treated prostates harvested after a two month recovery period without DES revealed ductal dilatation (ectasia) and persistent distortion of ductal architecture. Ectasia was most severe in the proximal ducts near the urethra and in prostates 17 weeks or older at the end of one month of DES treatment. The clinical consequences of early alteration of prostatic ductal architecture and development are potentially deleterious, as men who were prenatally exposed to DES may be at increased risk for the development of prostatic disease.

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INTRODUCTION

From 1948 to 1971, 4-6 million pregnant women and their unborn children were exposed to diethylstilbestrol (DES) to prevent spontaneous abortion [DHEW, 1978]. The hazards of this prenatal estrogen were first recognized in 1970 when Herbst et. al. reported seven cases of vaginal adenocarcinoma in young daughters of women who were treated during pregnancy with DES [Herbst and Scully, 1970; Herbst et al., 1971]. Since then an extensive literature has developed on the malignant and nonmalignant consequences in women prenatally exposed to DES. The long-term effects of transplacental DES exposure in the human male, however, are not yet known. Several clinical studies have cited increased genitourinary structural anomalies in adult men who were exposed to DES in utero [Bibbo et al., 1977; Cosgrove et al., 1977; Gill et al., 1979; Whitehead and Leiter, 1981]. These include an increased incidence of epididymal cysts, hypotrophic testes, hypoplastic penis, urethral stenosis, hypospadias, cryptorchidism and varicocele. Additionally, semen analysis has revealed changes in spermatazoa of DES-treated men [Bibbo et al., 1977; Gill et al., 1979; Whitehead and Leiter, 1981]. However, other clinical studies have failed to detect any significant differences between DES-treated men and age-matched controls [Andonian and Kessler, 1979; Leary et al., 1984].

Autopsy studies of human male infants have suggested a causal relationship between maternal DES therapy and interference with normal prostatic and testicular development [Driscoll and Taylor, 1980; Hoefnagel, 1976; Kaplan, 1959]. Driscoll and Taylor reported abnormalities of the utricle, dilated prostatic ducts, and squamous metaplasia in over 90% of the prostates of human infants exposed *in utero* to DES [Driscoll and Taylor, 1980]. To develop a relevant experimental model for this human condition, Sugimura et. al. grew human fetal prostates derived from abortus specimens for one month under the renal capsule of intact athymic male nude mice that were either untreated or treated with DES [Sugimura et al., 1988]. Of the 17 specimens exposed to

DES, 15 showed severe squamous metaplasia and distention of the urethra and prostatic utricle, while 13 had metaplastic changes in the proximal ducts. In contrast, only two of the control prostates exhibited even mild squamous metaplasia of the proximal ducts.

Prostatic squamous metaplasia occurs spontaneously in normal human fetuses beginning approximately at 22 weeks of gestation, presumably due to the high levels of maternal estrogens late in pregnancy [Andrews, 1951; Brody and Goldman, 1940; Driscoll and Taylor, 1980; Jakobsen, 1951; Mawhinney and Neubauer, 1979; Parkes, 1954; Sharpey-Schafer and Zuckerman, 1941; Zondek and Zondek, 1975]. However, both the incidence and degree of prostatic squamous metaplasia are variable in the normal population with spontaneous regression occurring within one month after birth [Andrews, 1951; Driscoll and Taylor, 1980]. This study was undertaken to determine if the metaplastic effects of DES on the human fetal prostate seen after one month of continuous DES exposure are likewise reversible or if there are long term persistent prostatic abnormalities associated with DES exposure of the human fetal prostate.

METHODS AND MATERIALS

Urogenital sinuses and prostatic rudiments from human fetuses 6-22 weeks of gestational age, as assessed by heel-to-toe measurements [Robboy et al., 1982], were obtained after therapeutic abortion performed for reasons unrelated to this study. Specimens were processed in a manner similar to that reported previously [Sugimura et al., 1988]. After delivery, the abortus specimens were placed in normal saline and transported to the laboratory where prostatic rudiments and urogenital sinuses were dissected from the surrounding tissues. Prostatic rudiments were then bisected sagittally into right and left halves with the veruomontanum as a midline landmark. One half of the fetal prostate was grafted aseptically under the renal capsule of an intact, untreated (control) male Balb/c nude mouse, while the contralateral half was similarly grafted into a

DES-treated host. Drug exposure was accomplished by subcutaneous implantation of a 25- to 30 mg pellet of DES, whose release rate in the host is approximately 60 μg/kg body weight/day [Kirkman and Bacon, 1952; Robboy et al., 1982], a dosage comparable to that given therapeutically to pregnant women [Smith et al., 1946].

Human fetal prostatic specimens were grown for one month in either untreated or DES-treated male hosts and then harvested and retransplanted under the renal capsule of intact, untreated male nude mice. In some cases biopsies of the one month grafts were taken before retransplantation. Both the control and DES-treated prostates were then allowed to grow without exogenous DES exposure for an additional two months. The grafts were then harvested, fixed in Bouin's fixative, embedded in paraffin, serially sectioned at 6 μm, and stained with hemotoxylin and eosin.

Histological examination was performed on 21 prostates for which both the control and DES halves were available. An additional seven specimens were histologically prepared and examined immediately after the first month of DES treatment. Squamous metaplasia was scored as follows: (-) = absent; (+) = mild, present but less than four layers thick; (++) = moderate, more than four layers thick but without luminal occlusion; and (+++) = severe, complete occlusion and distention of the lumen. Ductal dilatation (ectasia) was graded as follows: (+) = moderate, the normal linear appearance of the ducts was preserved but the lumina were dilated; (++) = severe, dilatation and cystic changes that dramatically altered ductal architecture.

RESULTS

Twenty one matched specimens were recovered after one month of initial growth in DES-treated hosts plus an additional two months of growth in untreated hosts (Table 1). Because the fetal age at the time of prostatic procurement ranged from 6-22 weeks, the corresponding gestational age equivalents of the specimens at the time of harvest

ranged from 18-34 weeks. Both the control and DES-treated human prostatic grafts became revascularized and grew significantly, exhibiting a many-fold increase in volume during the 3-month period in the nude mice. Nineteen of the control specimens exhibited normal ductal morphogenesis (fig. 1). The number of epithelial outgrowths from the urethra, the percentage of cellular cords that developed lumina, and the complexity of the branching pattern increased with the advancing age of the fetal prostate at harvest. Since normal prostatic buds emerge from the urethra at 9-11 weeks after conception [Johnson, 1920; Kellokumpu-Lehtonen, 1980; Lowsley, 1912], the youngest specimens obtained at 6-9 weeks of gestational age probably did not have prostatic buds at the time of grafting. At harvest these young specimens exhibited numerous solid prostatic ducts distally and the beginning of ductal canalization proximally (figs. 2 and 3). Stratified columnar epithelium lined the canalized portion of the proximal ducts. The epithelium of the distal solid cords consisted of three to five cell layers. Within the solid cords most of the epithelial cells were round, unpolorized, and had small hyperchromatic nuclei (fig. 3). Before ductal canalization, the epithelial cells became polarized. The long axis of the oval nuclei became aligned perpendicularly to the basement membrane with polarization first appearing in the basal layer of cells along the basement membrane (fig. 4). The more centrally situated cells became oriented at later stages just before a small central lumen appeared (fig. 5). In older specimens, solid prostatic ducts (presumably present at the time of grafting) elongated, branched and differentiated into tubuloacinar glands (fig. 6). As the ducts canalized and matured they became lined by a bi-layered columnar epithelium. Since epithelial polarization and lumen formation occurred in a wave of differentiation from the urethra to the distal ducts, ducts at different stages of differentiation were found in the each section. Small patches of squamous metaplasia were observed in the proximal ducts of one control specimen. One control specimen did not contain prostatic tissue, and one contained dilated, cystic ducts.

Of the seven DES-treated specimens harvested at one month, six contained

abnormal, irregularly shaped ducts distended with metaplastic cells. Severe squamous metaplasia was observed in the urethra and proximal ducts (figs. 7A and B). Mild to moderate squamous metaplasia was present in the distal ducts and acini (fig. 8) of all six grafts that were 17 weeks or older at the time of harvest. Ductal distortion was more severe in proximal ducts with areas of more normal ductal architecture distally (figs. 7 versus 8). The one specimen that did not exhibit any metaplastic or cystic changes was ten weeks of gestational age when harvested. These findings are consistent with those of Sugimura et. al. who observed that DES-induced effects are age-dependent with squamous metaplasia of the proximal ducts found only in specimens grown to a gestational age equivalent of $\geq$ 17 weeks [Sugimura et al., 1988].

When squamous metaplasia was observed, its distribution and severity within a specimen varied along the duct and between ducts. In some histological sections there appeared to be universal metaplastic transformation of the epithelium, particularly on the urethra and the proximal ducts closest to the urethra. However, careful examination of the serial sections revealed that focal areas of squamous metaplasia often originated in a duct from multiple, discrete sites (fig. 9). Proliferation of squames from one area displaced normal epithelium and filled the entire lumen for some distance. Adjacent epithelial cells appeared to be morphologically normal with gradations between columnar cells and squamous cells in the same duct.

In many of the 21 DES-treated specimens (Table 1) that were allowed to recover in normal male hosts for two additional months, squamous metaplasia often persisted, the incidence and severity varying directly with the increasing age of the prostatic grafts and proximity to the urethra. However, severe metaplasia was never observed in any region. Mild to moderate urethral squamous metaplasia persisted in 14 specimens and was most often found in the older specimens. Squamous metaplasia of the proximal ducts was observed only in those specimens $\geq$ 17 weeks of gestational age at the end of their one month of DES exposure. In only 2 specimens, which were also 17 weeks or older, did

metaplastic changes persist in the distal ducts. These observations reinforce the concept that the effects of DES are age-dependent. Once DES was withdrawn and the prostates were allowed to grow in a physiologic androgenic environment provided by the nude mouse host, squamous metaplasia regressed in varying degrees. Squamous metaplasia was observed in only 1 control specimen grown for the full 3 month period in untreated hosts.

The most striking morphological change seen in the DES-treated prostates harvested after a two-month recovery period was the presence of ductal ectasia in 14 of 21 specimens. Of those that were 17 weeks or older at the end of DES exposure, 10 of 11 exhibited ductal ectasia. Five of these 11 specimens had cystic dilitations and grossly abnormal ductal architecture (fig.10). Side buddings and distal ducts had a greater diameter than some of the proximal ducts. Cystic areas were often lined with a single layer of flattened, heterogeneous epithelial cells. Areas of nearly normal columnar or cuboidal cells were adjacent to apolar cells with scant cytoplasm. Atypical patterns of canalization could be seen in the distal ducts and acini, including loss of cellular polarity, heterogeneous cell size and shape, and irregular, cystic lumina (fig.11). However, in all but the most severely affected specimens, areas of normal-appearing uncanalized distal ducts and acini could be found. Consistent with the distribution of squamous metaplasia, ductal ectasia (Table 2) was most severe proximally and tended to involve more of the distal ducts and acini in the older prostates (fig. 12). Ductal ectasia was observed in only 2/21 control specimens grown for the full 3 month period in untreated hosts.

DISCUSSION

In developing human prostates, squamous metaplasia induced by DES varies in severity according to both the gestational age during the time of DES exposure and the relative proximity of the affected area to the urethra [Sugimura et al., 1988]. At any

given gestational age, the prostatic urethra was the most severely affected. Focal metaplastic changes were observed in the proximal prostatic ducts with only mild metaplasia distally. Squamous metaplasia induced by estrogen is also known to differentially affect different lobes or zones of rodent and human prostates [Huggins and Webster, 1948; McNeal, 1983; Price and Williams-Ashman, 1961]. Multiple but discrete areas of epithelium exhibited metaplastic changes along prostatic ducts. Additionally, proximal ducts of prostatic specimens younger than seventeen weeks of gestational age at the end of DES exposure did not demonstrate squamous metaplasia. The temporal and spatial distribution of these metaplastic changes induced by exogenous DES may be related to the ontogeny and/or distribution of estrogen receptors in either the epithelium or the mesenchyme of the developing prostate. A similar age-dependence of estrogen responsiveness was observed for developing human vagina [Cunha et al., 1987]. Immunocytochemical localization of the estrogen receptors in the rat and dog prostate has demonstrated that their location is consistent with the areas of histological change induced by estrogen administration. The concentration of the estrogen receptors was greatest in the stroma of the prostatic urethra and the proximal prostatic ducts. Estrogen receptors were not found in distal ducts or acinar epithelium [Cooke et al., 1991; Schulze and Barrack, 1987]. The ontogeny of estrogen receptors may explain why younger human fetal prostates failed to demonstrate effects of exogenous DES. A nonrandom spatial distribution of estrogen receptors with a higher expression in the proximal ductal regions would account for both the spatial distribution and focal nature of the squamous metaplastic changes induced by DES in the developing human prostate.

The age dependency and spatial development of ductal ectasia seen in the DES-treated prostates after 2 months of recovery are consistent with the initial distribution and severity of squamous metaplasia seen after one month of DES exposure. Significant and consistent ectasia occurred in proximal prostatic ducts of specimens seventeen weeks or older at the end of one month of DES treatment. Additionally, ectasia and cystic

dilatation was more severe proximally with preservation of apparently normal uncanalized distal ducts, although more of the total duct became involved in the older specimens. The ectactic process appeared to be initiated by the proliferation of squamous cells into the lumen. When the squamous metaplasia was severe, the build-up of sloughed cells distorted and distended ductal shape. Once the estrogen stimulus was removed, the sloughed squames degenerated within the distended ducts, but these ducts did not return to a normal architectural pattern. Possible ductal obstruction may further increase cystic dilatation. Mild changes are probably reversible, as seen in the normal human fetus in which normal prostatic metaplasia develops around 22 weeks of gestation but resolves within one month after birth. In this experimental model of DES exposure, more severe cystic abnormalities may be irreversible and certainly do not show evidence of improvement after two months of recovery in normal male hosts.

The clinical significance of these findings can only be speculative, but the ramifications of early alteration of prostatic ductal architecture and development with possible persistence of the abnormalities into adulthood are potentially deleterious. Exposure of the developing rat prostate to exogenous estrogen results in permanent suppression of prostatic growth and reduction in responsiveness to androgen in adulthood [Chung and MacFadden, 1980; Chung and Ferland-Raymond, 1975; Naslund and Coffey, 1986; Rajfer and Coffey, 1979; Rajfer and Coffey, 1978]. This is associated with changes in the cellular composition of the prostate and a reduction in androgen receptor levels [Prins, 1992; Prins et al., 1993]. In rats and mice treated prenatally with exogenous estradiol or DES, metaplastic epithelial changes persist long after cessation of estrogen treatment [Arai, 1970; Arai et al., 1978; Arai et al., 1977; McLachlan et al., 1975]. At 270 days after estrogen treatment in rats, papillary epithelial outgrowths markedly disorganized the normal prostatic urethral transitional cell epithelium; and at 20-21 months after perinatal DES administration, 2 of 11 rats developed solid tumors of the prostate resembling highly invasive squamous cell carcinoma [Arai, 1970; Arai et al.,

1978; Arai et al., 1977]. McLachlan et. al. reported that mice treated prenatally with DES developed prostatic adenocarcinoma and adenomatous hyperplasia 20 to 26 months after exposure [McLachlan et al., 1975]. Isaacs has proposed that the initiation and/or the promotion of carcinogenesis in the human prostate may be related to its basic ductal structure with regard to its ability to secrete, transport, and reabsorb various materials across and out of the ductal lumina [Isaacs, 1983]. Isaacs makes the argument that intermittent stasis of prostatic secretion leads to formation of corpora amylacea. Subsequent calcification of these bodies are significantly more prevalent in populations with high incidence of prostatic cancer. Since chemical carcinogens can enter prostatic fluid from the blood, and since some of the basic components of prostatic fluid may bind chemical carcinogens [Forsgren et al., 1979; Smith et al., 1977a; Smith et al., 1977b], stasis of prostatic secretions in grossly malformed ducts would provide a longer time for interaction of luminal cells and carcinogens.

McLachlan has expressed concern over the potential teratogenic and carcinogenic effects of the many estrogenic compounds found in our environment, including insecticides, drugs and naturally occurring substances found in foodstuffs and breastmilk [McLachlan, 1981a; McLachlan, 1985]. These effects are of particular importance in developing tissues since the differentiation and proliferative responses elicited by estrogen in target tissues have been shown to be reversible in adults but are often persistent and irreversible in younger animals [Bern and Talamantes, 1981; McLachlan, 1981b]. Although human males are naturally exposed to high levels of maternal estrogens during gestation [Fraenkel and Papanicolaou, 1938; Parkes, 1954], the cumulative effect of maternal estrogens, supplemental exogenous estrogens (e.g. DES) during critical periods of fetal development, and environmental exposure to estrogens before and after birth will at some level reach the threshold for deleterious biological consequences in men.

The incidence of prostatic adenocarcinoma is 120,000 new cases per year, usually

in men over the age of 50 [Chiarodo, 1991]. Men who were prenatally exposed to DES are now approximately 23 to 48-years-old. Within the next 10-20 years many of these men will be at risk for the development of benign and malignant prostatic neoplasms. Since the DES sons will soon be approaching the age when prostatic pathology is usually expressed, and since the long-term deleterious effects of DES are still unknown in men (but well documented in animals), it will be important to follow the DES sons carefully for the development of prostatic disease.

Table 1

Squamous Metaplasia in Human Embryonic Prostates Grown in Untreated and Diethylstilbestrol (DES)-treated Athymic Nude Mice

Specimen	Heel-Toe Length (mm)	Gestational Age Equivalent (Wk)¶			Control			DES*		
		t_0	t_1	t_2	Urethra	Proximal Ducts	Distal Ducts	Urethra	Proximal Ducts	Distal Ducts
Four Week Specimens§										
1	4	6	10	18	-	-	-	-	-	-
2†	17	13	17	25	-	-	-	++	++	+
3	22	14	18	26	-	-	-	+++	+++	++
4	27	16	20	28		No Ducts		+++	+++	+++
5†	28	16	20	28	-	-	-	+++	+++	++
6	34	18	22	30	-	-	-	+++	+++	+
7	45	22	26	34	-	-	-	+++	+++	++
Twelve Week Specimens§										
1	6	8	12	28	-	-	-	-	-	-
2	7	9	13	21		No Prostate		+	-	-
3	8	9	13	21	-	-	-		No Prostate	
4	8.5	10	14	22	-	-	-	+	-	-
5	9	10	14	22	-	-	-	+	-	-
6	9	10	14	22	-	-	-	-	-	-
7	11	11	15	23	-	-	-	-	-	-
8	13	11	15	23	-	-	-	-	-	-
9	14	12	16	24	-	-	-	+	-	-
10	14	12	16	24	-	-	-	-	-	-
11	17	13	17	25	-	-	-	++	++	+
12	20	14	18	26	-	-	-	+	-	-
13	23	15	19	27	-	-	-	-	-	-
14	24	15	19	27	-	-	-	++	+	-
15	25	15	19	27	-	-	-	+	-	-
16	25	15	19	27	-	-	-	+	+	-
17	25	15	19	27	-	-	-	+	++	-
18	28	16	20	30	-	+	-	+	+	-
19	31	17	21	31	-	-	-	+	+	-
20	36	19	23	31	-	-	-	+	-	-
21	39	19	23	31	-	-	-	++	++	+

Symbols:

* - = normal, no squamous metaplasia; +, ++, +++ = squamous metaplasia (+ = mild, ++ = moderate, +++ = severe; *see text*)

¶ t_0 = time of implantation, t_1 = time of harvest at 4 weeks' growth, t_2 = time of harvest at 12 weeks' growth

§ 4 week specimens were grown for 4 weeks and not reimplanted. 12 week specimens were grown for 4 weeks in either control or DES-treated hosts and then reimplanted and grown for an additional 8 weeks in a normal male host.

† Specimen nos. 2 and 5 were sectioned at four weeks for histology with the remainder being reimplanted and harvested at twelve weeks. These correspond to nos. 11 and 18, respectively, of the twelve week specimens.

Table 2

Ductal Ectasia in Human Embryonic Prostates Grown in Untreated and Diethylstilbestrol (DES)-treated Athymic Nude Mice

Specimen	Heel-Toe Length (mm)	Gestational Age Equivalent (Wk)¶			Control		DES*	
					Ectasia		Ectasia	
		t_0	t_1	t_2	Proximal Ducts	Distal Ducts	Proximal Ducts	Distal Ducts
Four Week Specimens§								
1	4	6	10	18	-	-	-	-
2	17	13	17	25	-	-	++	++
3	22	14	18	26	-	-	++	+
4	27	16	20	28	No Ducts		++	+
5	28	16	20	28	-	-	++	++
6	34	18	22	30	-	-	++	+
7	45	22	26	34	-	-	++	+
Twelve Week Specimens§								
1	6	8	12	28	-	-	-	-
2	7	9	13	21	No Prostate		+	-
3	8	9	13	21	-	-	No Prostate	
4	8.5	10	14	22	-	-	-	-
5	9	10	14	22	-	-	-	-
6	9	10	14	22	-	-	+	-
7	11	11	15	23	++	++	++	++
8	13	11	15	23	-	-	-	-
9	14	12	16	24	-	-	+	-
10	14	12	16	24	-	-	-	-
11	17	13	17	25	-	-	++	+
12	20	14	18	26	-	-	-	-
13	23	15	19	27	-	-	+	+
14	24	15	19	27	-	-	++	-
15	25	15	19	27	-	-	+	-
16	25	15	19	27	-	-	+	+
17	25	15	19	27	+	-	+	-
18	28	16	20	30	-	-	++	+
19	31	17	21	31	-	-	++	-
20	36	19	23	31	-	-	++	++
21	39	19	23	31	-	-	+	++

Symbols:

* - = normal, no ectasia; +, ++ = ectasia (+ = moderate, ++ = severe; *see text*)

¶ t_0 = time of implantation, t_1 = time of harvest at 4 weeks' growth, t_2 = time of harvest at 12 weeks' growth

§ 4 week specimens were grown for 4 weeks and not reimplanted. 12 week specimens were grown for 4 weeks in either control or DES-treated hosts and then reimplanted and grown for an additional 8 weeks in a normal male host.

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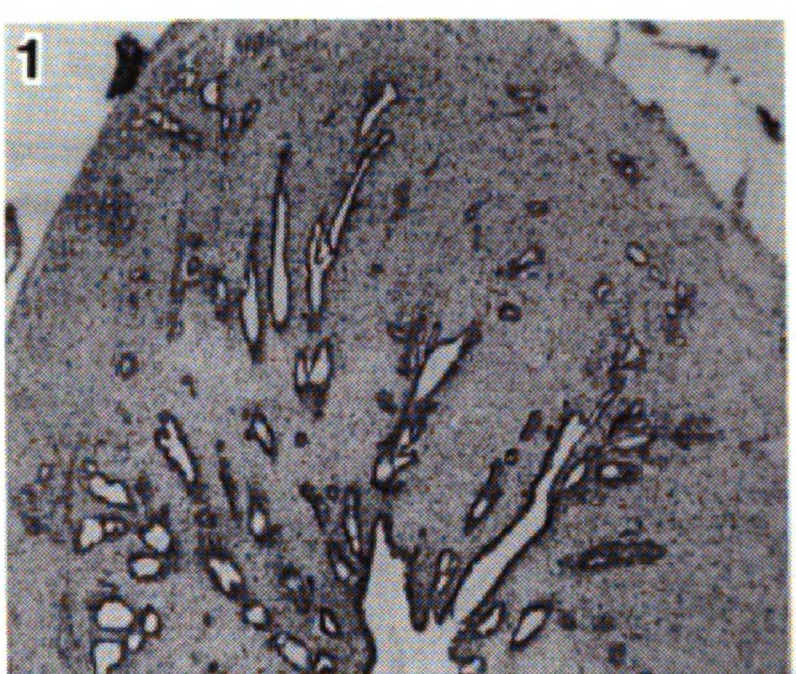
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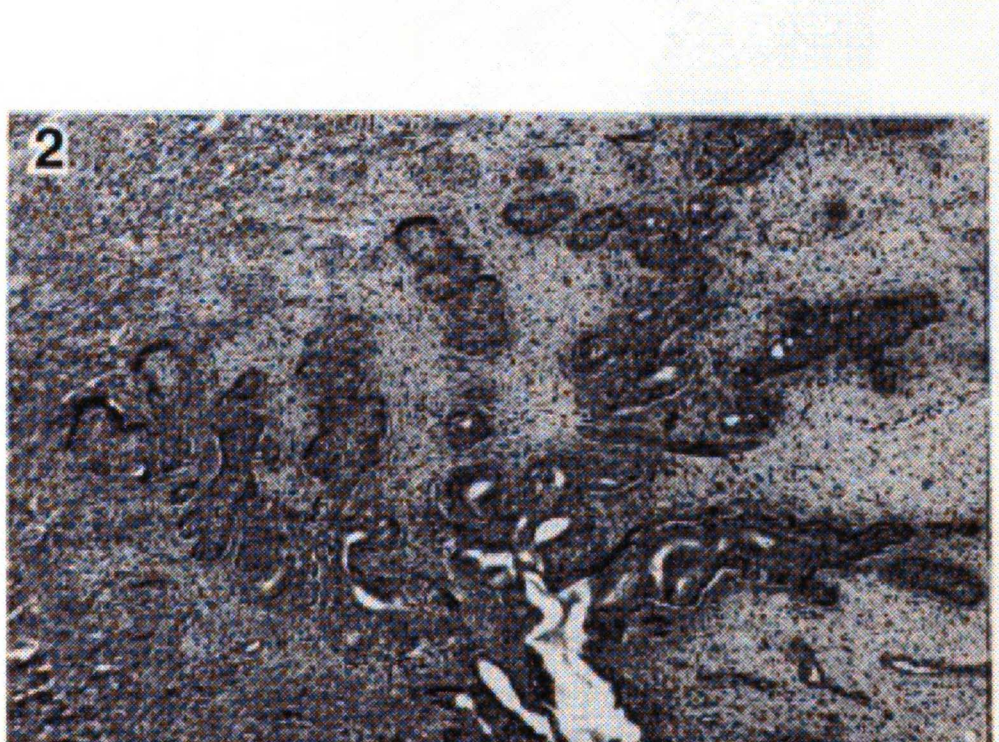
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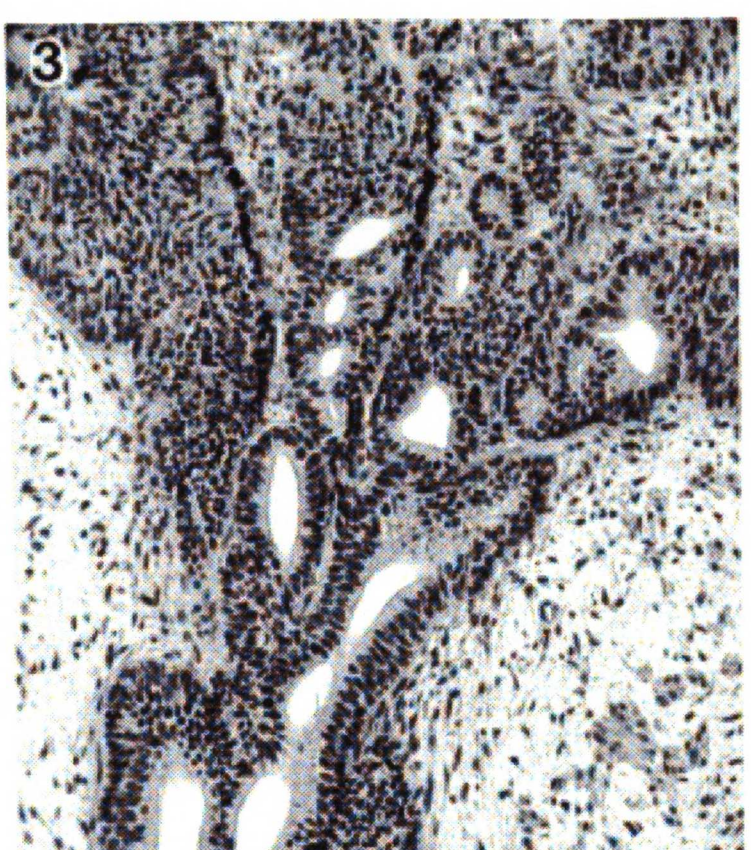
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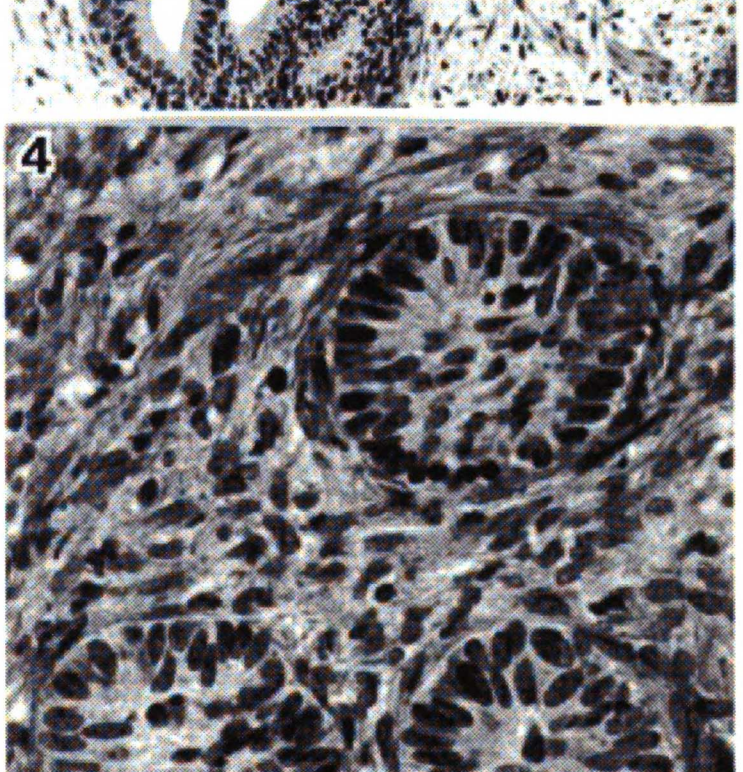
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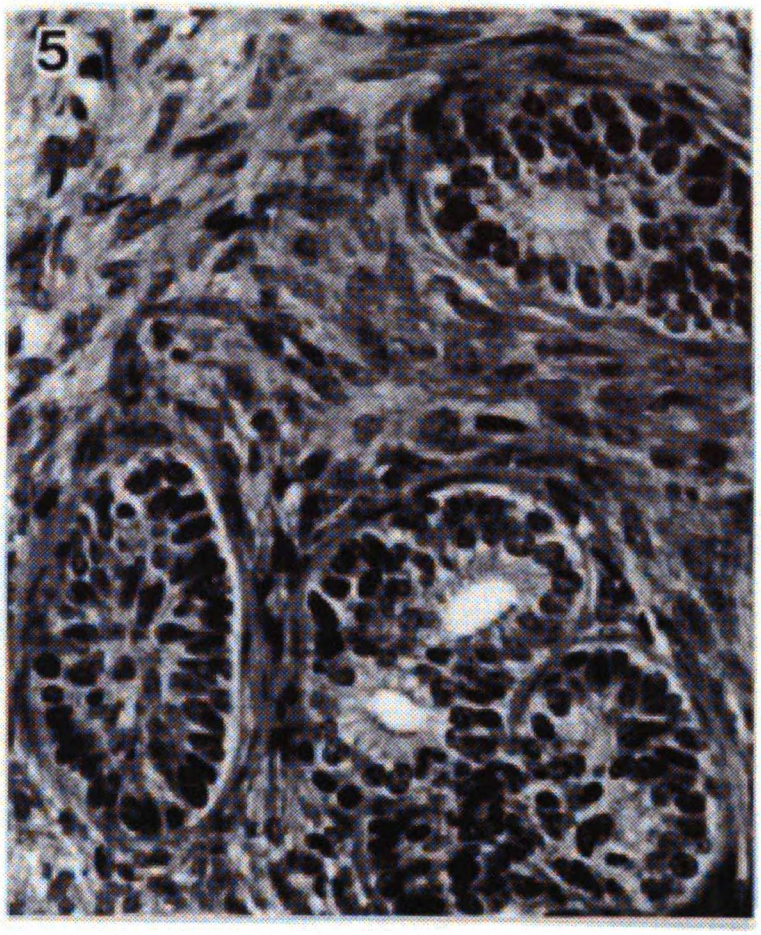
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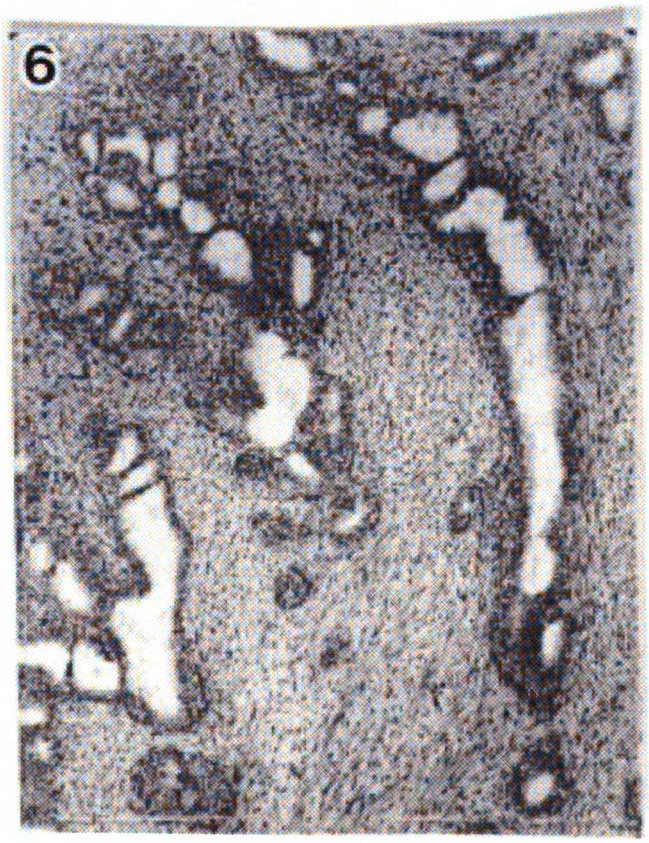
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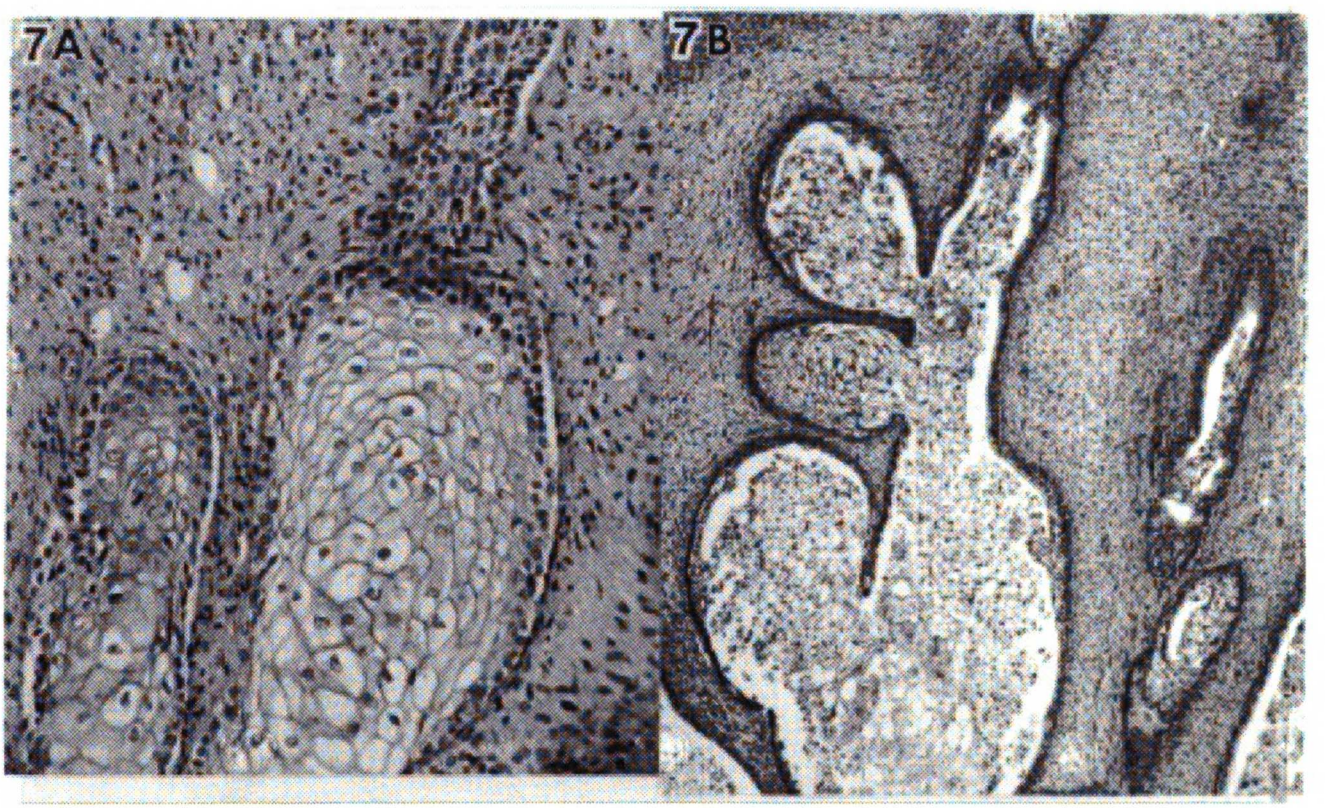
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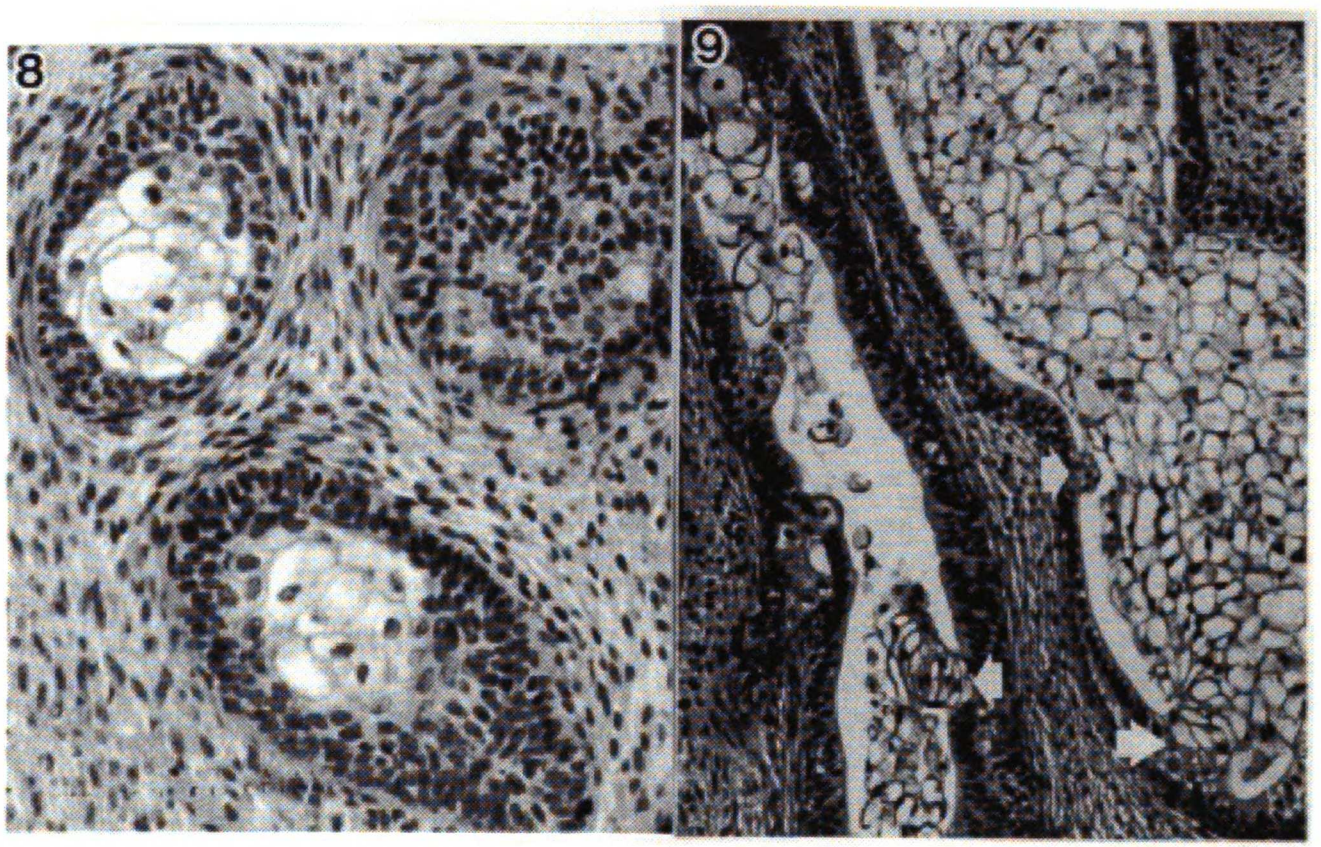
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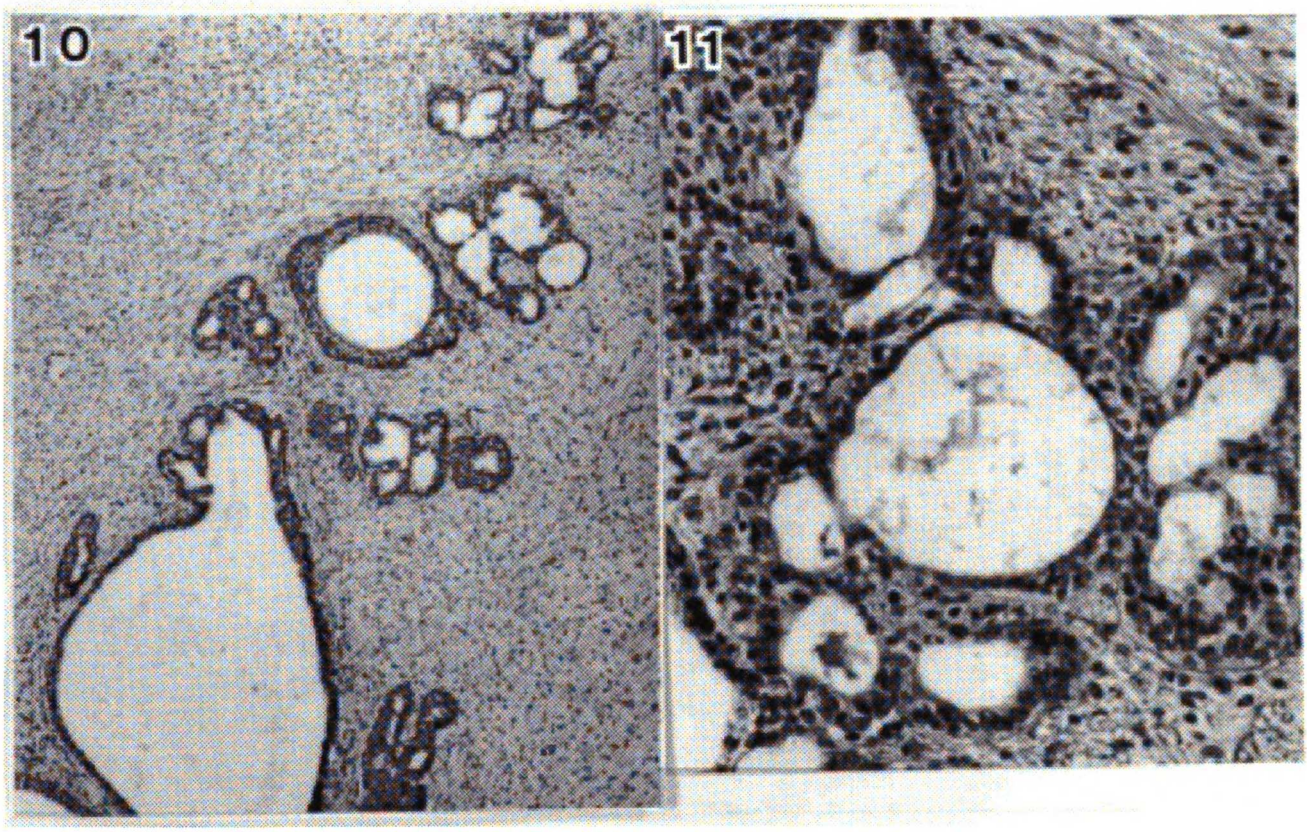
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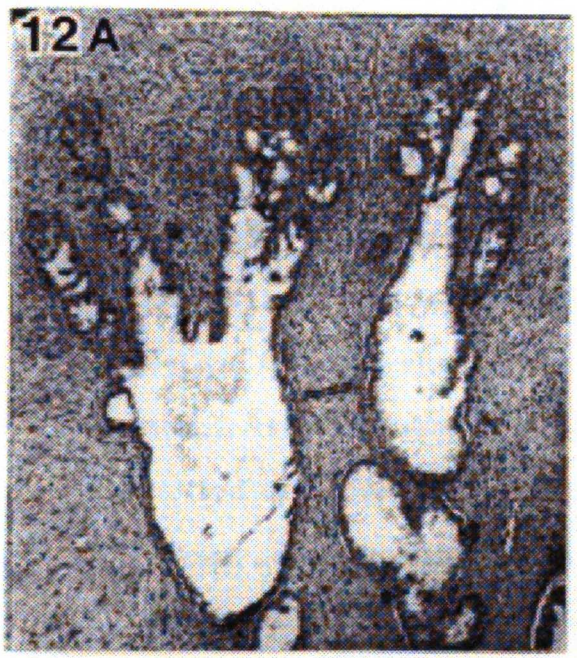
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EPILOGUE

The most common inquiry that has been posed to me is the relationship and/or relevance my research has to my clinical specialty. At first thought, it seems to be an important question, after all , my research involves the developing urogenital tract and my clinical specialty is in periodontics. However, if one examines some of the specifics involved with pursuing a research question as well as some of the mechanisms involved in the developing urogenital tract and the oral cavity, there exists a close relationship and strong relevance between my clinical specialty and my research. The following will be my perspective on the research that I have performed and the relevance it has to clinical periodontics.

The process of posing an hypothesis and testing it are somewhat universal and independent of a particular field. The gathering of information as a foundation for generating a question is similar, the only obstacle I have had is in learning some of the specifics, such as anatomy of an organ system, that does not translate well between systems. As to the testing of an hypothesis, the techniques involved are very similar. I have had extensive experience in cell/organ culture, tissue recombinations, micro dissection, and histological processing. These are not techniques that are unique to a particular field. Moreover, the preparing of a manuscript, including photography and application of statistics, is another exercise that has equal relevance between disciplines. Therefore, it is not surprising that an individual can learn the scientific process from an area that is not directly related to his/her clinical specialty.

I have alluded to a difficulty I have had in combining two disciplines. This is a real obstacle in that it required some extra time to familiarize myself with the specifics of each field. However, there exists some strong similarities in the mechanisms of development and differentiation between the disciplines. I will discuss these similarities from three inter-related perspectives: 1) The mechanism of reciprocal epithelial-mesenchymal interactions, 2) The role of growth factors in development and

differentiation, and 3) The role of the extracellular matrix (ECM).

The urogenital tract [Cunha, 1976] and oral cavity both undergo epithelial-mesenchymal interactions during development. The developing tooth begins differentiation as oral epithelium thickens, it then invaginates and grows into the underlying mesenchyme [Mina, 1987 , Kollar, 1983, 1991, Slavkin, 1991, Thesleff, 1991]. Moreover, in recombination experiments it has been shown that at days 9-12 in the rodent model, the oral epithelium exerts an instructive influence on the mesenchyme that begins the differentiation into a specific tooth pattern. Similar recombination experiments have shown that at later stages of development, there is a shift in instructive capacity to the mesenchyme. The exact mechanisms involved in this process are not completely understood and are quite complex. My interest in tooth development, however, is of a very specific area of the tooth. The differentiation of dental papilla mesenchyme into odontoblasts and oral epithelium into ameloblasts is not nearly as relevant to the field of periodontics as the differentiation of dental papilla mesenchyme into cementoblasts. There is currently much research in the field of regeneration of the periodontium [Ripamonti, 1994]. The focus of this research is aimed primarily at the regeneration of bone, secondarily at the periodontal ligament, and lastly the cementum. My bias is that the real difficulty in regeneration of the periodontium exists in the unpredictability of regenerating new cementum. Cementum is the slowest growing tissue of the three mentioned, and without it, repair and not regeneration occurs. Therefore, understanding what controls the growth of cementoblasts and deposition of cementum is a central question.

It is not understood, however, what controls the differentiation of cementoblasts. Is it from the oral epithelium/ameloblasts? Is it from the dental papillae/odontoblasts? If it is from the dental papilla, what other systems develop by having one mesenchymal cell instruct another mesenchymal cell to differentiate? Lastly, does the differentiation of cementoblasts in the fetus have application to the regeneration of cementum in the

adult?(i.e. is regeneration in an adult a wound healing event, or is it related to the differentiation precursor/stem cells?) These are all relevant and interesting questions that share the mechanism of epithelial-mesenchymal interactions that exists in both the developing oral cavity and urogenital tract.

Numerous growth factors have been implicated as important in the developing tooth and periodontium. Heikinheimo implicates TGF ß2 as a diffusible growth factor that could be important in a paracrine fashion that leads to the terminal differentiation of ameloblasts [Heikinheimo, 1993]. The mRNA of TGF ß2 was localized in the mesenchyme, but the protein was localized in the enamel organ during a stage that coincided with the terminal differentiation of ameloblasts. Others have examined the potential roles of TGF ß 1-3 and found that there are both unique and similar biological activities between the subtypes [Chai, 1994]. Meckel's cartilage development appears to be regulated by TGF ß 1 and 3, and TGF ß 2 seems to regulate tooth morphogenesis. The latter result being in agreement with Heikinheimo. In all of these experiments, however, *in vitro* systems failed to reproduce the results of tissue recombinations or *in vivo* development. Many of these authors concluded that the coordinated sequence of events that exists *in vivo* are not understood and can therefore not be reproduced *in vitro* (i.e. the importance of cell-cell and cell-matrix interactions).

Some very interesting work with growth factors has identified BMP-4 as an important signaling molecule in tooth development [Vainio, 1993]. Experiments have been done identifying BMP-4 expression during the period in which a dental epithelium has an instructive influence upon a non-dental neural crest derived mesenchyme. When the subsequent shift in instructive capacity goes from the epithelium to the mesenchyme, there is a parallel shift in the expression of BMP-4 from the epithelium to the mesenchyme. Their *in vitro* system included agarose beads as a means for locally delivering the BMP, and as in the aforementioned studies, there was not complete *in vivo* reproduction. However, their conclusion is that it is minimally interesting that there is a

shift in BMP-4 expression that coincides with a tissue's capacity for instructive induction, and more likely that there is an important role for BMP-4 in tooth development.

This same group has recently published some articles describing the presence of a particular heparin-binding growth factor called Midkine [Mitsiadis, 1995, 1995] that is localized to the cell surface of tissues undergoing epithelial-mesenchymal interactions. It alone does not seem to be stimulatory to cells in culture, but they hypothesize that it may have a regulatory function and works in conjunction with a cell surface ECM receptor. That particular ECM receptor is Syndecan, and they have also shown that it is present in the dental mesenchyme prior to tooth development [Vainio, 1992]. When the epithelium begins to migrate down during the early stages of tooth bud formation, Tenasin , an ECM glycoprotein that interacts with the cell surface and other ECM proteins, appears in the mesenchyme, and at later stages of development the two are colocalized. This is apparently common to other developing organ systems, but the exact roles for each have not been described.

During development, there seems to be a complex coordination between the epithelium and the mesenchyme that involves the cell surface, the ECM and growth factors. It may not be possible to separate each with its own influence because they may be interdependent. For example, a possible scenario could be that a diffusible factor(s) exert an influence only during a period in which certain cells of the ECM are brought in close approximation or direct contact with the cell surface, and any or all of these interactions can regulate the overall process. However, this scenario does not even address the potential influence of the cytoskeleton in growth, development and differentiation of cells or tissues. So while great strides have been made, and there is a great deal known about the development of many particular organ systems, the reality is that there is much still to learn. It is my opinion that understanding the particular organ system is not as important as understanding the concepts that are common to all. I strongly feel that although the particular organ system I have studied is not relevant to my

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