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Stimulation and inhibition of fibroblast subpopulations by phenytoin and phenytoin metabolites: pathogenetic role in gingival enlargement

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Abstract

Functional heterogeneity exists among fibroblasts in gingiva. There is remarkable variation in the protein synthetic activities, the proliferative capacities and the drug-response potentials of various subpopulations of such cells. These functional differences may play a role in the pathogenesis of phenytoin-induced gingival enlargement, as subpopulation mixtures are altered by conditions within the connective tissue milieu. This could result from stimulation of a subpopulation(s) characterized by elevated collagen synthesis or, alternatively, from inhibition of a subpopulation(s) characterized by low growth and synthetic potential.

Phenytoin and its metabolic breakdown products are present in significant quantity in the gingivae of phenytoin-treated patients. Data collected in our laboratory and by other investigators indicate that direct stimulatory or inhibitory action of phenytoin or a metabolite upon gingival fibroblast subpopulations is a factor in the pathogenesis of phenytoin-induced gingival enlargement.

Experimental data indicate that rapidly-dividing cell subpopulations are sensitive to phenytoin, while quiescent subpopulations are not.[1,2] The major metabolite of phenytoin in man is 5-para-hydroxyphenyl-5-phenylhydantoin (pHPPH). Addition of pHPPH to quiescent human gingival fibroblasts did not alter protein synthetic rates, nor was proliferation enhanced, nor were any cells killed by the treatment. However, pHPPH added to rapidly proliferating cells caused significant inhibition of replication in some strains.

The turnover rate of protein and collagen in gingiva is significantly higher than in other connective tissue sites,[3] reflecting cells in a highly active state. This may explain the particular sensitivity of gingivae to the action of phenytoin and/or its metabolite(s).

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Introduction

The purpose of this paper is twofold: One, to review the histopathology of phenytoin (PHT)-induced gingival enlargement and two, to present a fresh concept of the pathogenesis of this lesion, which has eluded elucidation despite 40 years of clinical and scientific effort. The history of the lesion and a summary of current work recently appeared.[4]

Histopathology

Since 1939, when Kimball[50] first noted gingival overgrowth as a side effect of long-term PHT therapy, numerous histopathological investigations of the lesion using the light microscopy and conventional staining method have appeared.[5-36] Several reports of transmission electron microscopic observations have also appeared in the world literature.[25,37,38,39,40] It was reported 25 years ago that the earliest detectable change was dilatation and engorgement of capillaries subjacent to the junctional epithelium,[15,41] resulting in hyperemia and edema soon after the initiation of PHT therapy.[42] Subjective evaluations noted increases in the number of capillaries and accelerated leukocyte transmigration.[16]

Concurrent with today's knowledge of the behavior of the periodontal soft tissues in health and disease, it is evident that investigators attempting years ago to microscopically characterize the earliest pathological alterations in PHT-induced gingival overgrowth, were actually describing what is now known as the "initial lesion" of inflammatory gingival and periodontal disease.[43] While, on the basis of numerous

investigations,[44,45,46,47,48,49] it appears that the host inflammatory response is indeed one factor in the multifactorial pathogenesis of PHT-induced enlargement, the classic capillary vasculitis and leukocytic infiltration of gingival tissue is neither peculiar to, nor pathognomonic for, this drug-related oral problem.

Two additional factors should be recognized in this regard: First, the patient who begins taking PHT often does so because he has experienced his first grand mal seizure, an event which is charged with emotional impact and which often institutes a weeks- or months-long period of stressful adjustment to a new and somewhat frightening fact of life. Second, until the patient's drug resonse is ascertained and the daily dose properly regulated, he is likely to experience PHT-induced CNS adverse effects such as lethargy or drowsiness. Taken together, these two factors — emotional stress and CNS side effects — are quite likely to effect the patient's oral hygiene habits. It has been amply demonstrated[51] that clinical and histological manifestations of gingival inflammation, the "initial lesion," will occur after only a week or so of inadequate oral hygiene.

The clinical classification of PHT gingival overgrowth as fibrous type, inflammatory type or combined type[52] is also a convenient descriptive system for the histopathological appearance of the mature lesion. The inflammatory type of PHT lesion of long standing is characterized by the presence of numerous host defense cells, with plasma cells predominating in most cases. In addition, there is often pronounced dilation of vessels, whose endothelial cells exhibit large nuclei with relatively basophilic cytoplasm which might reflect an active metabolic state.[16] The round cell infiltrate often replaces almost completely the collagenous connective tissue. Immunoglobulins may be present within plasma cells as well as extravascularly; this phenomenon was observed many years ago and was associated with the appearance of "pyronin-positive bodies."[25,53,16,54,55] These were once believed to be pathognomonic for the PHT lesion, but are now recognized as but another feature of the established lesion of inflammatory gingival and periodontal disease.[56] The presence of these "bodies" also gave impetus to the erroneous suggestion that PHT gingival overgrowth is an immunological disorder based on antigen-antibody reaction.[34]

In summary, the inflammatory-type PHT lesion does not exhibit any histopathological features which are pathognomonic for it. A pathologist without knowledge that the specimen on his microscope stage was from a patient taking PHT regularly would be likely to read out a histological diagnosis of chronic inflammatory gingival or periodontal disease.[57]

The "pure" PHT-induced gingival enlargement is the fibrous-type lesion. While it is true that even gingivae which appear clinically uninflamed almost invariably exhibit histologic evidence of low-grade inflammation, in the fibrous-type PHT lesion there is essentially none of the inflammatory cell accumulation and loss of collagen typical of the inflammatory-type lesion. In microtome sections of mature fibrous PHT lesions (Figure 1), the epithelium is acanthotic and keratinized to greater or lesser degree,[5,7] although true hyperkeratosis is only rarely observed.[9] Rete pegs often penetrate deep into the subjacent stroma[58] as cells of the stratum basale proliferate and are thrown up into folds. Increased mitotic activity in basal layer cells of human PHT-overgrowth gingiva has been reported.[7,59] Alterations within the stratum spinosum are not infrequently observed, as cells exhibit various levels of degeneration, most commonly manifested by the formation of vacuoles in association with cell nuclei. Intercellular "bridges" of the spinous layer appear also to undergo degenerative changes, and a larger than normal percentage of cells in this layer are undergoing mitosis.[60] The nature and significance of these epithelial alterations remain unknown. The question of possible epithelium-connective tissue interplay has recently been revived.[61,62,58,63]

Epithelial acanthosis may be a regular feature of the fibrous-type PHT lesion, but it is not pathognomonic and is not responsible for the clinically obvious increase in gingival dimension; this results from expansion of the connective tissue compartment. In the more initial stages of lesion development, routing H α E staining of specimens reveals numerous fibroblasts[21] which have been described as "small, with a lack of cytoplasmic basophilia."[15] Somewhat later, col-

Figure 1. Low power photomicrograph of trichrome (Gomori)-stained section through severe fibrous-type phenytoin-induced gingival overgrowth. Keratinized oral epithelium exhibits long rete pegs penetrating deep into the subjacent connective tissue stroma. A very mild round cell infiltrate is seen. Massive accumulation of collagenous connective tissue within the gingiva propria. (See cover of this issue.)

lagen accumulation has been observed. Kasai and Tanimoto,[22] for example, described a diffuse accumulation of fuchsin-affinity amorphous substance which they believed to correspond to collagen precursors. A rich network of "oxytalan fibers," now believed to represent precursor molecules and reticulin fibers which are stained with aldehyde fuchsin after reaction with peracetic acid,[64,65] was observed immediately subjacent to the epithelium.[66] Only a few investigators have attempted to identify the earliest characterisitcs of *developing* enlargement; most studies were performed by means of subjective evaluation of biopsies from mature overgrowth lesions of long standing.

Until recently, there was confusion concerning the nature of the expansion of the connective tissue component: some investigators[67,68,9,10,14,16,33] claimed, on the basis of light microscopic observations, that PHT-induced gingival overgrowth represents classical fibrosis, i.e., an accumulation of collagen fiber bundles to form a tissue characterized by relative acellularity. Indeed, in extreme cases the submucosal connective tissues are filled with heavy bundles of collagenous fibers which, when appropriately stained, appear to fill the entire submucosa. Heavy fibers are often observed in apparent intimate contact with the basement membrane subjacent to the epithelium.

On the other hand, other investigators[69] believed that increasing *cell numbers* account for the increase in gingival size, thus lending credence to the term dilantin *hyperplasia.* Many reports based upon subjective histological observations have supported the idea that fibroblast proliferation subsequent to PHT ingestion leads to an increase in the fibroblast component.[70,15,71,72,73,21,40,74,75,35]

This apparently dichotomous situation was given clarity recently with the publication of an objective, quantitative histologic and morphometric investigation.[17] The authors measured fibroblast density and collagen content in clinically noninflamed gingivae from patients exhibiting PHT-induced gingival overgrowth and analogous tissue from normal individuals. The fibroblast-to-collagen ratio was identical in gingival tissues from both sources (Table 1). Thus, mature, fibrous-type PHT-induced gingival overgrowth is an example of a connective tissue lesion characterized by redundant tissue of apparently normal cell and fiber composition.[76,77] This situation pre-requires abnormally large numbers of fibroblasts and abnormally large amounts of fibroblast products (e.g., collagen) *per oral cavity.* It appears that at some point in the development of the mature lesion, normal cellular growth control is lost and an abnormally high level of fibroblast mitotic activity occurs. Many investigators have speculated that PHT may act as a mitogenic agent, inducing rapid cell division in resident connective tissue cells, and evoking a true cellular hyperplasia, albeit a transient one.[15,72,21,78,79,80,81,82,35] The current experimental evidence concerning this possibility will be presented in detail later in this paper.

Of the five electron microscopic investigations of PHT-induced gingival overgrowth published to date, not one is in the English language (three are in German,[25,37,38] one in Japanese,[39] and one in Spanish).[40] These reports disagree in their findings. One investigator claims that the collagen accumulated in connective tissue in PHT gingival overgrowth is immature, and that this is a consequence of rapid connective tissue proliferation stemming from relatively undifferentiated connective tissue cells.[37] In contrast, other investigators observed that collagen microfibril configuration and distribution were normal, and noted that the resident fibroblasts possessed a cytoplasm rich in rough endoplasmic reticulum and free ribosomes, indicating highly differentiated and specialized cells. It is clear that further investigation employing the electron microscope is warranted, and work of this nature — both on excised tissue and on gingival fibroblasts *in vitro* — is in progress.[83,84]

Direct action of phenytoin gingival cells/ tissues

Of the many theories concerning etiological mechanisms that have been proposed, the one that has gained substantiation from several sides, and which lends itself readily to further investigation, is the possibility that PHT gingival overgrowth is due to direct action of the drug or one of its metabolites upon resident cell populations within the gingival tissues.[85-92] One report suggested that the severity of gingival enlargement is associated with higher PHT levels in human gingival tissue,[93] and the drug has been detected in oral mucosa, gingiva, salivary glands and saliva of man and various experimental animals.[94,93,95,96,97,98,99,100,92]

In a 1964 pilot study,[101] a statistically significant correlation was found between PHT content of saliva and occurrence and severity of gingival enlargement, but this finding has not been substantiated in three studies of larger epileptic populations by independent investigators.[146,44,93] While some have suggested that saliva-borne PHT may indeed elicit gingival overgrowth, others are quick to point out that the most frequent sites of gingival involvement — mandibular anterior, maxillary anterior and maxillary posterior regions (in descending order of frequency) — are not near the major salivary duct orifices. This argument is supported by the work of Meyer,[102] who demonstrated that there is very little exchange of oral fluid contents among various regions of the oral cavity. While it is likely that the majority of the PHT detected in the gingival connective tissue reaches the site via the circulating blood, saliva-borne drug may also contribute to the total, assuming the drug can traverse the

epithelial barrier. This possibility was mentioned 25 years ago by Van der Kwast,[34] and has recently been demonstrated in an experimental animal model (rabbit) by Steinberg and his co-workers.[103,104,105,90,108] It is not surprising that the relatively low molecular weight, lipid-soluble PHT molecule can enter the gingival sulcus, penetrate the junctional epithelium, and come to rest within the subepithelial connective tissue. Previous reports indicate that considerably larger molecules possess this capability.[107-109] In addition, it has been demonstrated that elevated levels of inflammation correlate positively with the inward penetration of particles into and through the gingival sulcular epithelium and subjacent connective tissue.[110]

Dental plaque absorbs PHT from saliva,[90] accumulates it, and may thus play a dual role in the pathogenesis of gum overgrowth by eliciting inflammation which subsequently enhances the passage of PHT from oral fluid, and from the plaque itself through the sulcular epithelial tissues. Furthermore, it was recently demonstrated[111] that human gingival fibroblasts have the capacity to metabolize phenytoin to hydroxylated by-products (Figure 2).

If it is assumed that PHT and its metabolites are deposited within gingival connective tissue, the potential effects these compounds might have on the proliferative capacities or the protein synthetic activities of gingival fibroblasts are obvious. It is not surprising

Table 1. Quantitation of the fibroblast component in normal and PHT-enlarged human gingiva by direct method.[1]

Specimen	Measured nuclear length*	Corrected nuclear length	Apparent nuclei per field	Apparent nuclei per mm²	Actual nuclei per mm²
Normal gingival tissue					
A	17.6 ± 4.9	22.3	17.3 ± 4.5	623	114.1
B	16.2 ± 3.8	20.5	13.9 ± 3.6	500	98.0
C	16.5 ± 4.1	20.9	14.9 ± 4.2	536	103.5
D	17.1 ± 3.8	21.7	8.8 ± 3.2	317	59.4
E	15.3 ± 2.9	19.4	10.2 ± 2.6	367	75.2
F	15.8 ± 3.4	20.0	18.2 ± 5.8	655	131.0
G	16.2 ± 4.5	20.5	13.6 ± 2.9	490	96.1
H	16.5 ± 3.7	20.9	10.2 ± 2.7	367	70.8
I	15.7 ± 3.5	19.9	9.2 ± 2.2	331	66.5
J	16.5 ± 4.2	20.8	12.1 ± 3.4	436	84.5
x ± SD		20.7 ± 0.9	12.8 ± 3.3	462 ± 119	89.9 ± 22.7
PHT-enlarged tissue					
A	16.7 ± 4.3	21.1	10.1 ± 3.1	364	69.7
B	16.7 ± 4.7	21.1	12.1 ± 4.1	436	83.5
C	17.2 ± 5.3	21.8	16.1 ± 5.2	580	108.2
D	16.9 ± 3.9	21.0	15.0 ± 5.7	540	103.8
E	15.6 ± 3.5	19.4	11.9 ± 3.9	428	87.7
F	15.8 ± 4.2	20.0	7.4 ± 2.7	266	53.2
G	16.6 ± 4.7	21.0	15.1 ± 4.8	544	104.6
H	15.3 ± 3.8	19.4	11.7 ± 4.0	421	86.3
I	16.1 ± 4.4	20.4	8.5 ± 2.7	306	60.2
J	16.1 ± 3.9	20.4	10.6 ± 3.1	382	75.2
K	15.4 ± 3.6	19.5	13.0 ± 1.9	468	95.5
L	15.3 ± 4.4	19.4	11.4 ± 2.0	410	78.3
x ± SD		204 ± 0.8	11.9 ± 2.6	429 ± 95	83.9 ± 17.5

* Zeiss micrometer ocular. Magnification = x 400. x ± SD for 100 measurements.

Abercrombie (Anat. Record *94:* 239, 1946): for thin sections, corrected nuclear length = mean apparent nuclear length 0.79.

x ± SD for 30 counting fields.

mm² = 35 counting fields at x 400 magnification.

Abercrombie (1946); P = (A) (M/L+M), where P = actual number of nuclei per mm², A = apparent number of nuclei per mm²; M = section thickness in micra; L = mean corrected nuclear length in micra.

[1]From Hassell, T., Page, R. and Lindhe, J.: *Archs Oral Biol,* 23:381-384, 1978. Reproduced with permission of Permamon Press, Oxford.

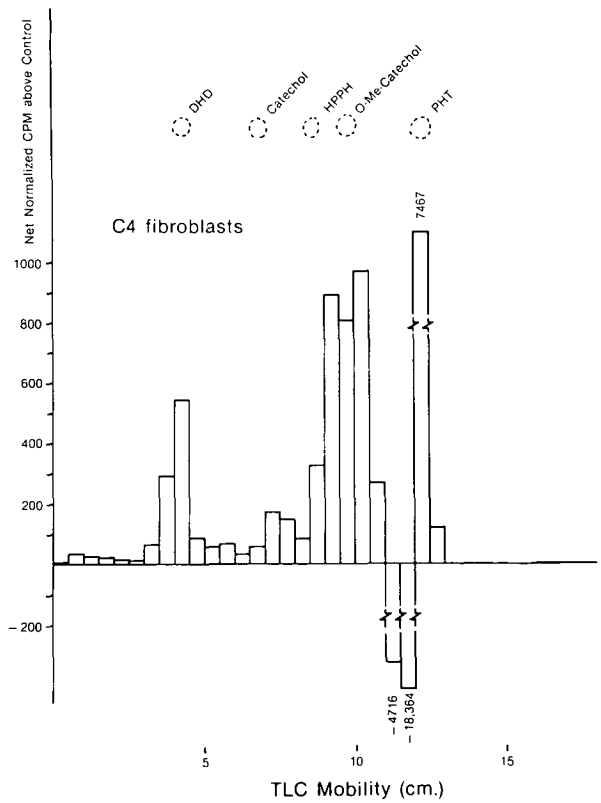

Figure 2. Evidence of phenytoin metabolism by gingival fibroblasts *in vitro*. Nonconfluent cultures in MEM medium containing 10% fetal calf serum were pulsed for five days with 2 uCi of [4-^{14}C]-5,5-diphenylhydantoin. Cultures were freeze-thawed 3X, then harvested by scraping. Pooled cells and medium were pre-extracted with $CHCl_3$ and the PHT metabolite extracted into ethyl acetate, then evaporated to dryness under N_2 gas. Residue was taken up in microliter quantities of methanol, spotted on Gelman TLC (thin layer chromatography) plates, and developed versus known standards. The resultant chromatograms were sliced at 5 mm intervals and the slices subjected to liquid scintillation counting. Results were normalized for total net CPM above controls (^{14}C-PHT pulse in cell-free medium). In the C4 cell strain depicted, significant PHT-dihydrodiol was observed.

that numerous investigators during the past 20 years have attempted to test these putative effects in *in vitro* culture of various cell types. Unfortunately, these investigative attempts have not yet conclusively proven that PHT effects collagen synthesis by direct interaction with fibroblasts. The not insignificant technical problems associated with this experimental approach were recently reviewed in detail.[4] Furthermore, many factors — most of which cannot be evaluated in a cell culture system — likely play roles in the pathogenesis of the gingival lesion, e.g., modulation of the pituitary-adrenal cortex axis, inhibition of host immune system phenomena, persistent local irritation or antigenic stimulation of the tissue due to microbial deposits upon the teeth and within the gingival sulci, genetic susceptibility of the host, and regular intake of the drug for fairly long periods of time (six months or more). It is apparently the interplay among these factors which leads, with time, to development of enlarged gingivae.

However, there is a major conceptual difference between attempting to *recreate* this complex situation in the tissue culture dish (by adding PHT to culture medium, for example), and attempting to *capture* the already existent situation. If the fibroblasts within overgrown gingival tissue are, as a result of the circumstances enumerated above, abnormally active synthetically, it is reasonable to assume that this activity could be monitored by exposing freshly excised pieces of overgrown gingival connective tissue to radiolabelled amino acids in complete medium.[112,113,114] Preliminary investigations indicated that, in comparison to tissue bits from "normal" human gingiva, tissue from fibrous PHT-enlarged gingiva synthesizes elevated levels of protein and collagen *in vitro* in the absence of any PHT in the medium.[115]

Subsequently, fibroblasts were permitted to emigrate from primary explants of gingivae from a number of young PHT-treated epileptics exhibiting severe fibrous gingival overgrowth, from age-matched PHT-treated epileptics who had never experienced gingival enlargement, and from many nonepileptic individuals. All cells were grown and passaged in medium containing no phenytoin. When the protein and collagen synthetic activities of these various cell strains were measured, again using incorporation of radioactive amino acids as the parameter, it was found that cells from overgrown gingiva were still producing about twice as much protein per cell when compared to "normal" cells or cells from nonovergrown gingiva.[116-118] Furthermore, in responder cells from overgrown tissue a much larger portion of the protein synthesized was collagen (ca. 20 versus ca. 10% in normal and nonresponder cells). It must be emphasized that this experiment was performed using cells which had been passaged 3-10 times in the absence of PHT. This demonstrates the stability of the peculiar phenotype of the fibroblasts derived from overgrown gingivae, i.e., the phenotype is propagable throughout many cell doublings *in vitro*. We are dealing, then, with a cell which is apparently permanently biochemically "different" from its morphologically identical normal gingival counterparts.

The mechanism by which the interplay among PHT and the other etiological factors induces such an effect on fibroblasts remains obscure. But if it is assumed that all factors other than PHT are predisposing factors, and not direct causative ones, the hypothesis which appears most compatible with the observations to date is one of *selection*, by the unique conditions existing within the affected tissues, of an unusual fibroblast *subpopulation*. A small portion of

the fibroblasts normally present in gingiva may inherently possess the properties of elevated protein synthesis and unusually high collagen production. A unique combination of conditions, plus PHT or one of its metabolites, existing in the gingival tissues of some individuals may lead, through selective growth pressures, to amplification of the population size of the cells with these properties. This particular subpopulation of fibroblasts is thus induced to become the predominant cell type in the tissue. When a biopsy of this tissue is obtained and placed into culture, the fibroblasts which emigrate are, for the most part, cells of the peculiar subpopulation. Daughter cells quite naturally maintain the phenotype of their antecedents.

As depicted schematically in Figure 3, this hypothesis presupposes the existence of several or many phenotypically distinct and different subpopulations of fibroblasts within the gingivae (and other connective tissues) of normal individuals. Conceptually, the unique features of a given normal connective tissue would, at least in part, be the result and reflection of normally functioning subpopulation mixtures. It seems entirely plausible that in chronic diseases of the periodontium, a combination of etiologic factors modulates these subpopulation mixtures, resulting, with time, in the presence of abnormal subpopulation mixtures of otherwise normally functioning cells. Consequently, changes in the matrix, which we recognize as disease, occur. A fair amount of data, reviewed below, has accumulated from many laboratories which supports not only the concept of functional heterogeneity of fibroblast populations in normal tissue but also the apparent participation of such peculiar cell subpopulations in various disease states.

Some properties of connective tissues can best be accounted for by postulating the existence of heterogenous fibroblast subpopulations. For example, at least five genetically distinct collagen types have been identified, and the relative proportions of these vary greatly from one tissue to another. One might reasonably expect these to be produced by cells of different types (cf. the production of specific antibody by particular clones of lymphoid cells).

Recent *in vitro* work by Engel et al.,[119] using fluorescent antibody labeling of intracellular collagens of various types, indicates that within a mixed population of normal human gingival fibroblasts, some cells are producing only one type of collagen, while some other cells may produce more than one type (Figure 4). Previous studies by other investigators have also indicated that multiple genomes exist for different collagens and that one cell "type" may be capable of elaborating more than one species of collagen.[120,121,122]

In most body tissues, the turnover rate of connective tissue substance decreases with increasing age, but collagen turnover in the periodontal tissues and in healing wounds remains very high, even in adults.[123,124,125,3,126] These variations in collagen type, amount and

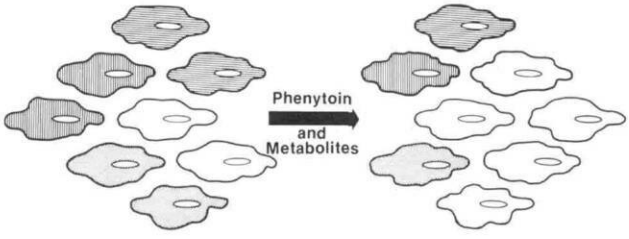

Figure 3. Schematic depiction of fibroblast subpopulation selection hypothesis. Several or many different types of fibroblasts are present within normal gingivae. Some of these cells may be predisposed by the action of secondary etiological factors (see text). In presence of PHT or a PHT metabolite, the "responder" fibroblast subpopulation is induced to become the predominant cell type in the tissue, as other subpopulations are inhibited, or as the responder type is stimulated.

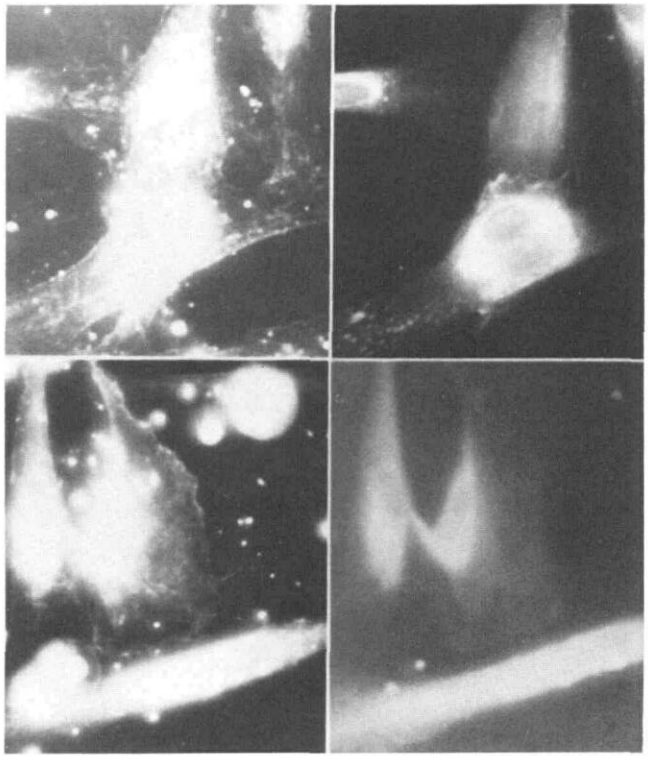

Figure 4. Darkfield and immunofluorescent photomicrographs of human gingival fibroblasts. (A) Darkfield of cells shown in 4B (upper left). (B) Cells stained with specific antibody to type I procollagen (upper right). Staining is most intense in the region around the nucleus. Note that one of the cells is negative for type I procollagen. (C) Darkfield of cells shown in 4D. (lower left). (D) Cells stained with specific antibody to type III procollagen (lower right). The staining is of much weaker intensity than seen with type I antibody, indicating that these cells contain less type III procollagen than type I procollagen. Magn. X400. Reproduced from Engel et al., *Archs Oral Biol* (in press, 1980), with permission of Pergamon Press, Oxford.

turnover time may be functions of the particular fibroblast subpopulations making up the tissue at a given point in time, rather than modulation of the activities of a single cell type. Another example, recently reported, is that the chain composition of collagens extracted from edentulous ridge connective tissue differs significantly from that of gingiva from dentulous individuals.[127]

Martin et al.,[128] isolated and propagated *clones* of cells in culture from a "mixed" population of human diploid fibroblasts derived from a single skin biopsy. Among the subclones, extensive epigenetic heterogeneity was noted with respect to replicative potential. Similarly, Milunsky et at.,[129] placed 1 mm fragments of connective tissue from a single foreskin into five separate culture dishes, nourished and incubated each dish identically, and performed enzyme assays (β-galactosidase, hexosminidase, β-glucuronidase, β-glucosidase and arylsulfatase A) on the fibroblasts which emigrated into each dish from the primary explant. They detected 60-500% variations in enzyme activities among cells from the five separate dishes (Table 2), indicating functional heterogeneity of fibroblasts from a single source. Kaufman et al.,[130] detected different patterns of testosterone metabolite accumulation in early-passage subcultures of skin fibroblasts developed simultaneously from single explants of one prepuce. This, too, reflects heritable heterogeneity of connective tissue cells. The testosterone metabolism patterns observed persist through serial culture of the clones to sensescence, thereby eliminating the possibility that they reflect functional disparities among individual fibroblasts based upon their variable replicative ages.[131,132]

Studying a strain of diploid human gingival fibroblasts derived from a normal, healthy, young, male donor, Ko et al.,[133,134] demonstrated that a particular "cellular hormone" (prostaglandin E_2) reacts *in vitro* with a subpopulation of approximately 45% of the cells to completely inhibit protein synthesis, DNA synthesis and cell growth, with no perceptible effects on the remaining cells. As a consequence, the prostaglandin-sensitive cells appear, with time, to become deleted from the parent population (Table 3).

When normal human gingival fibroblasts are exposed to fresh human serum, DNA synthesis is increased by 30-50% compared to identical cells exposed to heat-inactivated serum.[135] In addition, "suicide" experiments have been performed in which these same cells are maintained in fresh or heat-inactivated serum in the presence of bromodeoxyuridine, then treated with Hoechst 33258 bisbenzimidazole dye and exposed to light to preferentially kill ("suicide") cells which had incorporated the bromodeoxyuridine. After subsequent re-exposure of both groups of cells to fresh serum, the cells which had survived the suicide in the presence of heat-inactivated serum exhibited in-

Table 2. Lysosomal enzyme activities in cultured fibroblasts grown in quintuplicate from the same skin biopsy.[1]

		Enzyme Activities (n moles/mg protein)				
Cells		β-Glactosidase	Hexosaminidase	β-Glacuronidase	β-Glucosidase	Arylsulfatase A
Series 1	a	331	2047	189	25	18
	b	233	2325	85	12	10
	c	230	1875	96	14	8
	d	292	2546	106	20	11
Series 2	a	179	2306	210	21	42
	b	161	1982	141	19	34
	c	86	1849	179	8	19
	d	119	2176	129	11	30
Series 3	a	87	2668	182	27	31
	b	199	2359	161	26	24
	c	155	2153	147	19	13
	d	153	2051	149	30	23
	e	180	1828	150	24	24

Five primary fibroblast strains were derived from a single human foreskin, then 14 subcultures were obtained by trypsinization and re-seeding. The subcultures were harvested, and centrifuged at 600 x g and the pellet assayed for various enzyme activities by established methods after sonication (see reference 129 for details). Each value reported is the mean of triplicate assays; intrasample variation did not exceed 5-8%.

[1]Reproduced from Millunsky, A. et al., *Life Sci,* 11:1101, 1972, with permission of Pergamon Press, Oxford.

Table 3. Evidence for a prostaglandin-sensitive subpopulation of cells within a mixed culture of normal human gingival fibroblasts.

Medium supplemented with	Number of Labeled Nuclei	% Reduction
Nothing (control)	20	
10% Fetal calf serum (FCS)	1,459 ± 21	
10% FCS + 10^{-5}M Prostaglandin E_2	851 ± 104	42

1.5 x 10^5 serum-starved quiescent, synchronous, diploid, human gingival fibroblasts were seeded onto 22 mm^2 coverslips in 35 mm plastic petri dishes containing RPMI 1640 medium (GIBCO) without serum. After 2 hr incubation, cultures were made 10% in FCS to activate DNA synthesis, and some cultures received prostaglandin E_2 at 10^{-5}M. Cultures were pulse-labeled from hour 12 to hour 33 following serum activation by addition of 2 Ci/ml (^{3}H)-thymidine, washed by dipping five times in cold PBS, fixed in Bouin's solution for 20 minutes at room temperature, air dried, coated with nuclear track emulsion (Eastman Kodak NTB No. 2), developed in Eastman Kodak D19 and counterstained with hematoxylin and eosin. For each coverslip, the portion of labeled nuclei was determined by counting 2,000 cells in randomly selected microscopic fields. Data are presented as mean labeled nuclei (± SD) for triplicate cultures. Reproduced from Ko, S. D. et al., *Proc Natl Acad Sci,* 74:3429, 1977, with permission.

creased levels of DNA synthetic activity as compared to the cells which had died in the presence of fresh serum (Table 4). These results point toward the existence of a discrete subpopulation of fibroblasts which is susceptible to a mitogenic factor or factors present in fresh serum but absent in heat-inactivated serum.

It is also possible to demonstrate in cultures of fibroblasts derived from a single explant, that some cells are rapidly replicating while others are slow or non-replicating. In some instances, there are demonstrable morphological differences between the two populations, such as changes in nuclear size,[137] but in most instances these subpopulations can only be detected by autoradiography after a tritiated thymidine pulse.[138]

In studies by Felix and DeMars[136] of the X-chromosomal Lesch-Nyhan syndrome, fibroblast cultures from heterozygotes were shown to contain subpopulations of HG-PRT (hypoxathine-guanine phosphoribosyltransferase)-deficient cells that would grow out of colonies in the presence of 8-azaguanine.

It has been popularly assumed that one way to "synchronize" mixed cultures of fibroblasts, i.e., to inhibit further mitotic activity by trapping the cells in the G_1 or G_0 phase of the cell cycle, is to culture them in medium containing no serum. Usually, all cell division will cease after 24-48 hours of serum starvation. However, in a recent study of 18 different strains of

Table 4. Selective subpopulation killing of fibroblasts by BrdUrd-light treatment.[1]

Treatment I + BrdUrd	Treatment II	CPM*
Heated human serum (HHS)	No serum	374 ± 54
HHS	HHS	771 ± 87
HHS	FHS	4480 ± 377
Fresh human serum (FHS)	No serum	335 ± 34
FHS	HHS	699 ± 89
FHS	FHS	952 ± 74

* Reported as mean ± SD of ^{125}IdUrd incorporation for quadriplicate culture of normal human diploid gingival fibroblast. Quiescent (serum-deprived) cultures were activated to begin DNA synthesis by exposure to fresh or heat-inactivated (56°, 30 min) human serum in the presence of BrdUrd. Culture was then treated with Hoechst 33258 bisbenzimidazole dye and exposed to light. Afterwards, both groups (HHS and FHS) were re-exposed to fresh serum, and DNA synthesis determined by uptake of ^{125}I-UdR.

[1]From Korotzer, T. et al., *J Cell Physiol,* in press, 1980; with permission of Alan R. Liss, Inc., N.Y.

human gingival fibroblasts (Table 5), it was discovered that some cells continue to traverse the cell cycle, and to divide, even after 84 hours of total serum deprivation,[139] indicating that while mitogens in serum are required for cell proliferation in most fibroblast subpopulations, there exist other subpopulations which can continue to cycle along nicely without serum factors. In contrast, cultured scleroderma fibroblasts have increased sensitivity to biosynthetic stimulation by serum;[35] adding serum enhanced the basal rate of collagen synthesis by as much as 445% in the affected cells, but only 43% in normal skin fibroblasts.

These few examples demonstrate the strength of the experimental evidence for the existence of functionally different subpopulations of fibroblasts within normal connective tissue. The evidence continues to accumulate;[140-144] most recently, Smith and Whitney[145] reported that even the two daughter cells arising from a single fibroblast mitosis may differ by as many as eight population doublings (= 256-fold in the number of cells produced) in their ability to proliferate.

If the existence of functionally heterogeneous subpopulations of fibroblast is clear, the role of such subpopulation in disease pathogenesis — specifically with regard to the phenytoin-induced gingival lesion — is not. The proposed hypothesis is the existence of a resident subpopulation of gingival fibroblasts characterized by elevated levels of protein and collagen production which is in some way induced to become the predominent cell type in the tissue. But how does PHT, or one of its metabolic breakdown products, act upon one or more subpopulations of gingival fibro-

blasts, rendered susceptible by predisposing factors, to induce it (them) to become, with time, the predominant cell type in the gingiva? At least two possibilities are immediately apparent. First, the drug or a metabbolite may stimulate the cell type in question to proliferate, while having no mitogenic effect on other subpopulations. Alternatively, the drug or a by-product of it may be cytotoxic for those fibroblast subpopulations characterized by low synthetic activity or low proliferative capacity.

The question of possible PHT mitogenicity remains to be answered. Perusual of the cell culture literature is confusing. Until very recently, it was impossible to reconcile the inconsistent and contradictory results, largely due to the different methodologies employed, different drug concentrations and drug vehicles used, different cell types studied, etc. For example, PHT concentrations of up to 10,000 ug/ml have been tested[69] on fibroblast-like cells isolated from human tissues; in one test series, maximal stimulation of cell proliferation was reported at 200 ug PHT/ml of culture medium, which is 10 times the therapeutic serum level in humans. On the other hand, Naess[147] found that only 40-60 ug PHT/ml inhibited cell multiplication, while 80-100 ug/ml resulted in cell death. At 5-40 ug/ml, there was no stimulation of proliferation. Hassell and co-workers found that while 10 ug/ml did not stimulate the proliferation of any of 15 different strains of human gingival fibroblasts derived from normal individuals and from PHT responders and nonresponders, even 100 ug/ml did not kill these cells,[148,149] as shown in Table 6.

Likewise, Kasai and Yoshizumi[150] were unable to detect proliferative enhancement of human gingival cells with any concentration of PHT, although feline gingival cells were slightly stimulated in the presence of 1.7-3.3 ug/ml. Above 6 ug/ml, growth of all their cell strains was inhibited. Keith et al.,[151] detected no consistent mitogenic effect on PHT on WI-38 (human fetal lung) fibroblasts. Houck et al.,[152] studying human skin fibroblasts, detected stimulation of 36% by addition of PHT (2-10 ug/ml) to their culture medium. This was

Table 5. Susceptibility of 17 strains of human gingival fibroblasts to serum deprivation-induced quiescence.

Cell strain	Hours of Serum Deprivation					
	24	36	48	60	72	84
Normals						
N-1	2093 (338)	799 (131)	229 (41)	283 (41)	222 (43)	156 (24)
N-2	2610 (863)	553 (182)	186 (35)	123 (63)	633 (221)	417 (300)
N-4	146 (35)	154 (64)	37 (10)	1604 (364)	486 (364)	64 (21)
N-5	1643 (307)	849 (107)	112 (53)	59 (19)	299 (96)	64 (15)
N-6	4685 (640)	3750 (268)	1404 (170)	2747 (540)	2302 (121)	4904 (490)
N-7	2821 (334)	1718 (381)	1261 (119)	793 (92)	794 (83)	1383 (630)
Nonresponders						
NR-1	1562 (100)	1348 (235)	935 (151)	1268 (168)	1812 (297)	1747 (65)
NR-2	77 (21)	43 (1)	32 (10)	41 (13)	39 (15)	31 (3)
NR-4	664 (83)	798 (144)	127 (36)	121 (30)	148 (58)	178 (42)
NR-5	146 (43)	250 (64)	150 (42)	191 (53)	70 (10)	89 (12)
NR-6	1891 (438)	3091 (511)	1788 (391)	2123 (356)	3172 (475)	4481 (500)
Responders						
R-1	307 (57)	2097 (50)	840 (398)	852 (172)	275 (57)	151 (76)
R-2	1652 (165)	1202 (288)	968 (69)	1255 (246)	1234 (111)	1310 (153)
R-3	1558 (76)	1306 (168)	481 (72)	803 (82)	632 (139)	1042 (80)
R-4	1566 (261)	1662 (155)	649 (69)	1721 (83)	1467 (160)	1369 (123)
R-6	713 (158)	2845 (720)	1047 (109)	805 (45)	682 (276)	2120 (406)
R-7	1663 (132)	1122 (125)	271 (46)	748 (70)	702 (139)	1037 (151)

Human gingival fibroblasts were seeded at 5000 cells per micro test well in medium containing 2% fetal calf serum, and allowed to attach for 6 hr. Medium was then removed, the cell layer rinsed twice gently with Hanks basic salt solution, and serum-free medium added to each well. At intervals from 24 to 84 hours, triplicate wells were pulse-labeled with 0.5 uCi ^{125}I-UdR for 2 hr. Harvest was with 30% trypsin solution in EDTA buffer, using a SKATRON^{R} microharvester, and gamma counting was performed. Data are reported as mean ($\pm$ SD) for triplicate wells. In this system <200 CPM is considered quiescent. Note that nine cell strains did not achieve quiescence even after 84 hours of serum starvation.

manifested as a reduction in doubling time from 35 to 22 hours.

Intraperitoneal injection of PHT (3 mg/kg) altered the growth pattern and morphology of the tumor cells,[153] and significantly prolonged the life span of Ehrlich ascites tumor-bearing mice indicating a toxic effect on PHT on rapidly proliferating cells. Similarly, Benveniste and Bitar recently reported that log growth phase cultures of human fibroblasts from the overgrown gingiva of PHT-treated epileptics respond to culture medium containing 5 ug PHT/ml,[154] while contact-inhibited "quiescent" culture does not.[118]

Similar contradictory results are found in the literature with regard to the putative effects of PHT on protein and collagen synthesis by various types of fibroblast-like cells in culture. Benveniste and Bitar,[154] for example, reported that 5 ug PHT/ml of medium stimulated actively growing responder gingival fibroblasts to synthesize increased quantities of protein and to secrete an increased percentage of that protein as collagen. Similarly, Kasai and Hachimine[88] detected increases in *in vitro* collagen synthesis by feline and human gingival cells of 66 to 84%, respectively, after 11- to 14-day exposure to PHT at 1-5 ug/ml. Hassell et al,[1] detected no such synthetic enhancement in any of 15 strains of confluent human gingival cells exposed to PHT at a concentration of 5 ug/ml. Houck et al.,[131] also detected no increase in collagen production by skin fibroblasts when PHT was added to 2-20 ug/ml. PHT has been reported to augment collagen maturation in normal skin,[155] to accelerate gingival wound healing[156] and to strengthen scars.[81]

Table 6. ^{51}Cr-Release assay for evaluation of cell killing by PHT *in vitro*.

		PHT per ml					
Cell strain	SDS	Fresh Medium	2 ug	5 ug	10 ug	50 ug	100 ug
Normals							
N-1	2442*	635	532	550	535	548	537
N-2	751	258	243	259	239	280	224
N-4	1773	553	591	606	571	557	514
N-5	771	225	201	236	229	279	263
N-6	1420	510	507	492	458	503	461
N-7	1155	316	292	317	305	258	351
Nonresponders							
NR-1	1266	370	350	405	363	369	339
NR-2	843	213	181	193	222	216	186
NR-4	641	226	236	234	264	250	190
NR-5	1344	432	388	421	427	425	423
NR-6	1045	348	343	367	333	349	344
Responders							
R-1	698	181	188	224	296	195	198
R-2	614	183	154	183	174	184	165
R-3	721	153	155	160	—	161	189
R-4	978	278	276	270	269	265	252
R-5	1195	351	399	369	379	353	365
R-6	522	152	147	136	147	192	128

*Reported as mean counts for triplicate wells. Suspensions of 17 different strains of human gingival fibroblasts were exposed to 200 uCi of ^{51}Cr, seeded into microwells at 10^4 cells per well and allowed to attach for 16 hr. Then PHT at concentrations of 2-100 ug/ml was added, and incubation continued for an additional 24 hr period at 37C. Supernatant medium was then harvested and the amount of ^{51}Cr released was determined with a gamma counter. Sodium dodecylsulfate (SDS) was added to some wells as a positive control (to burst all cells and release all ^{51}Cr); negative control wells received fresh medium only. Not even the highest PHT concentration killed any strain of cells, as test well cpm were universally lower than negative control values.

The question of a possible role for PHT *metabolites* in the pathogenesis of gingival overgrowth is a recent one. In man, the major metabolite of PHT is 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH). It is found in blood, saliva and in the gingival tissues of PHT-treated epileptics. pHPPH administered orally to cats elicits gingival overgrowth that is clinically and histologically similar to PHT-induced lesions in man.[157,158] Furthermore, some gingival fibroblasts have the capacity to metabolize the parent drug to HPPH, apparently by way of a PHT-dihydrodiol intermediate (see above, Figure 2, and reference 111).

We have studied the possible stimulatory action of HPPH on human gingival fibroblasts *in vitro*. The compound did not stimulate protein or collagen synthesis in any of 15 different strains of cells from responder, nonresponder or normal individuals (Table 7). These negative results, while requiring substantiation by other investigators, indicate that HPPH is not mitogenic, and that stimulation by HPPH is probably not a factor in the pathogenesis of PHT-induced gingival overgrowth.

However, there is data accumulating to substantiate the possibility that the major PHT metabolite selects for a particular subpopulation of fibroblasts via its cytotoxicity for cells not characterized by elevated activity. For example, though pHPPH even at very high dosage levels (50-100 ug/ml) will not kill human gingival fibroblasts *in vitro* (Table 8, references 159, 149), pHPPH has been shown to slow proliferation of some strains of cultured gingival cells while not effecting other strains (Figure 5A-F). Furthermore, pHPPH is even more potent than the parent compound in inhibiting microtubular polymerization. The metabolite also inhibits completion of mitosis in cell culture,[160] eliciting an accumulation of cells apparently "stuck" in metaphase. This effect is similar to that of colchicine but, unlike colchicine effects, it is reversible.

Stavchansky and co-workers[162,163] reported that, *in vitro* (rat liver 9000 g supernatant) HPPH alters cellular metabolism of some type I compounds, e.g., hexobarbital, and type II compounds, e.g., zoxazolamine. Since the metabolism of these compounds is believed to involve binding to distinct sites on cytochrome P-

Table 7. Effect of pHPPH on protein synthesis by various strains of human gingival fibroblasts.

Strain	Control	+ Vehicle	+ HPPH
N-1	81,150 (5531)	71,228 (10878)	67,776 (9353)
N-2	103,313 (2724)	89,940 (6677)	87,828 (6470)
NR-4	68,091 (8052)	59,812 (1065)	58,273 (6307)
NR-5	63,646 (2898)	60,297 (7100)	56,356 (7257)
R-1	131,776 (1641)	130,117 (14197)	106,236 (10917)
R-4	109,420 (7798)	108,841 (5959)	102,430 (10581)

Confluent cultures of six human gingival fibroblast strains were pulse-labeled for 24 hr with 5 uCi/ml (^{3}H)-Proline. Cells and media were harvested together into dialysis casings and unincorporated label removed by dialysis. Liquid scintillation counting was performed to determine total protein synthetic activity, and data are reported as mean CPM (± SD) per 10^6 cells (cell number determined by Coulter counter). Control cultures were never exposed to drug; vehicle-treated controls received an appropriate pulse of ETOH. There were no statistically significant differences among the three groups, indicating pHPPH does not effect protein synthesis.

Table 8. ^{51}Cr-release assay for evaluation of cell killing by pHPPH *in vitro*.

		pHPPH per ml of culture medium					
Cell strain	SDS	Fresh Medium	1 ug	2	5	10	50
Normals							
N-2	24182*	5329	5233	5514	5480	5503	4906
N-1	33160	7029	6689	6989	6689	7181	7031
Nonresponders							
NR-4	24501	4825	4733	4699	4314	4700	4406
NR-5	23529	4585	4776	5052	4796	4945	4471
Responders							
R-4	28215	5491	5556	5660	5591	5818	5245
R-1+	5924	1591	1435	1528	1418	1455	1416

*Reported as mean counts for triplicate wells. Experimental protocol as in Table VI. Even the highest HPPH concentrations did not kill any strain of human gingival fibroblasts.

+ Separate run

450, these investigators' results suggest that HPPH alters binding, or binds, itself to cytochrome P-450.[161]

There have been many reports of *in vitro* and *in vivo* studies indicating that PHT itself also exerts cytotoxic effects on cells,[164,165,166,151,153,147,60] but some of these investigations require independent substantiation because of the technical uncertainties associated with cell culture work.[4] Phenytoin inhibits DNA synthesis by proliferating human lymphoctyes,[167] but not fibroblasts,[149] and also inhibits polymerization of isolated, purified microtubules.[168]

A peculiarity of gingival tissue is its ability to accumulate levels of PHT and PHT metabolites in excess of the concentrations found in serum and saliva. Although it has not yet been corroborated by independent investigators in a larger patient population, reciprocal relationships among gingival content of PHT, gingival content of HPPH and severity of overgrowth

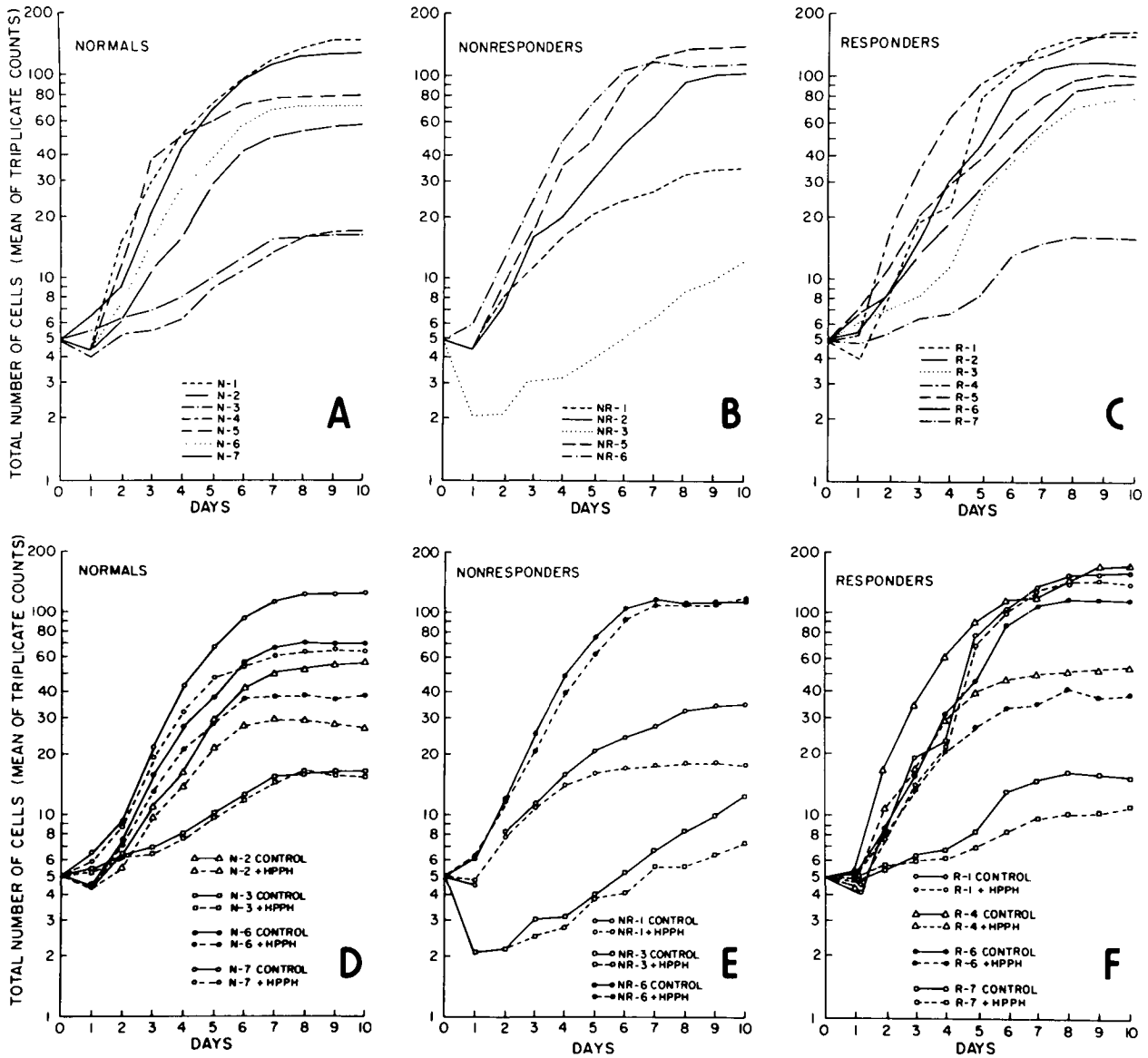

Figure 5. Growth curves for several strains of human gingival fibroblasts inuding cells from normal individuals as well as from PHT-responder and nonresponder epileptics. A large flask of cells was grown to early confluence in serum-containing medium, then harvested and seeded at 5000 cells per LinbroR well in 1.0 ml of medium. Twenty-four hours later, and daily for 10 days thereafter, three wells were harvested by trypsinization and total cell counts determined by Coulter counting. All wells were fed daily by removing 200μ1 of spent medium and adding 200μ1 of fresh, serum-containing medium. (A-C) Lag phase, log phase and postconfluent proliferative characteristics of 19 different fibroblast strains. There is considerable interstrain variation in growth potential. (D-C) In the presence of 5 ug/ml pHPPH, the growth rate was significantly inhibited in three out of four normal strains, two out of three nonresponder strains and three out of four responder strains of human gingival fibroblasts.

have been demonstrated in a pilot study. It is tempting to draw a parallel between these findings and the observation that other sites of PHT (and HPPH?) concentration — the brain, the liver, the adrenal glands — also correspond to sites of specific drug function, metabolism, and toxicity.

It appears, then, that the following factors are significant in the multifactorial pathogenesis of PHT-induced gingival overgrowth: (a) the existence of various subpopulations of cells which exhibit characteristic phenotypic peculiarities; (b) the accumulation of drug and/or metabolite(s) in the synthetically active gingival marginal tissue at concentrations beyond the typical somatic levels; and (c) demonstrated toxic effects of PHT and HPPH upon some connective tissue cells. If one or more subpopulations of gingival fibroblasts have the capacity to metabolize PHT, while other subpopulations do not, this may also play a role in the susceptibility or nonsusceptibility of some individuals to the drug-induced lesion.

Conclusion

In summary, the *fibroblast subpopulation selection hypothesis* is based upon the concept that functionally heterogenous subpopulations of cells exist within the gingivae and other connective tissues, and that the "normalcy" of a tissue is a reflection of a particular mixture, or "percent composition," of various subpopulations. Abnormality, i.e., pathosis, occurs when this composition is disturbed by endogenous or exogenous factors.

Conceptually, this hypothesis has quite a potential impact upon what have become some rather well-accepted concepts of disease pathogenesis. For example, the marked qualitative and quantitative alterations in connective tissues that occur in PHT-induced enlargement and in various other gingival and periodontal disorders are clearly important pathogenic consequences in the progress of such diseases. There has been considerable speculation that the etiology of these alterations is in some way related to some type of cellular injury. Thus, many have contended that cells which have been injured, for example, by components of the host inflammatory response or by exogenous cytotoxic substances, exhibit abnormal, compromised functions, and that this compromised cell function constitutes the primary factor in disease pathogenesis.[169,170,171] However, the demonstration of "disease phenotypes" which are genetically stable and propagable throughout many cell doublings in the absence of the purported etiologic factors supports the concept of subpopulation selection rather than cellular injury in the pathogenesis of connective tissue disorder. Such disease phenotypes have been demonstrated not only in PHT-induced gingival enlargement,[118] but also in inflammatory periodontal disease,[172] recessive dystrophic epidermolysis bullosa,[173] diabetes mellitus,[174] pretibial myxedema,[175] burn wounds,[125] Hurler's syndrome,[176] Marfan's syndrome,[177] scleroderma,[143,178,179,180,181] and rheumatoid arthritis.[182,183]

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