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VITAMIN A AUGMENTS COLLAGEN PRODUCTION BY CORNEAL ENDOTHELIAL CELLS

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SUMMARY: When isolated confluent corneal endothelial cells were cultured in delipidized serum, a marked reduction in collagen production was observed. Supplementation of such cultures with vitamin A as either retinol or retinoic acid at concentrations of 10^{-6}-10^{-7} M was capable of significantly increasing collagen production. In addition, when cultured in normal (non-delipidized) serum, both retinol and retinoic acid were capable of further increasing collagen production by corneal endothelial cells. Such augmentation of collagen production was relatively specific as total protein synthesis was not altered to the same extent, nor was it merely a reflection of changes in total cell number, as such cell numbers were similar in all treatment groups.

Production of extracellular matrix (ECM) components by endothelial cells appears to be markedly affected by microenvironmental influences (1-2). We have recently shown that exogenous fibronectin alters corneal endothelial cell phenotypic expression, e.g., cellular morphology and rate of collagen production (2). Such influences are of notable importance, as the product of such endothelial cells, i.e., the ECM is increasingly being recognized as playing a key role in human health and disease (3). Consequently, we have initiated a series of studies on the influence of essential nutrients, in this case vitamin A, upon endothelial cell phenotypic expression.

Despite reasonably intensive investigation of vitamin A for over half a century, the precise biochemical modes of action and the very basis of essentiality remain relatively unclear (4). It has long been recognized that adequate vitamin A was requisite for the differ-

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entiation of various tissues, particularly epithelium (5). Recent studies have shown that the precise level of vitamin A can markedly influence the state of differentiation in a large number of cell types, both in vivo and in vitro (6-9). A number of studies have indicated that vitamin A appears to have a central role in the synthesis of various glycoproteins (10-13) and glycosaminoglycans (14-16). Moreover, corneal endothelial cells have been demonstrated to have specific receptors for retinol (17) and retinol has been shown to influence the growth rate of bovine aortic endothelial cells (18). The eloquent studies of Fuchs and Green have shown that the precise level of vitamin A is capable of markedly modifying keratinocyte phenotypic expression, as reflected by alterations in parameters such as cellular motility, attachment, and production of different molecular species of keratin (9). It therefore seemed plausible that vitamin A might influence the level of collagen production by corneal endothelial cells. Indeed, it was observed that removal of the lipid fraction of the serum, which would contain the vitamin A, resulted in a sharp decrease in the level of collagen production. In contrast, supplementation with retinol (RET) and retinoic acid (RA) was associated with a three-fold increase in collagen production above those values observed in endothelial cells cultured in delipidized serum.

MATERIALS AND METHODS

Whole eyes were enucleated from 8-10 week old New Zealand White rabbits and the Descemet's Membrane (DM) and associated endothelial cells were isolated from underlying stroma (19). These endothelial cells were enzymatically dissociated from the DM and the cells were cultured in Dulbecco's modified Eagle's medium, 20% fetal bovine serum (FBS) and gentamicin (50 µg/ml). Confluent cultures were passaged (1:4) and secondary cultures were established. At confluency, cultures were incubated for either 48 or 96 hours in medium containing [^{3}H]-proline (12.5 µCi/ml), ascorbic acid (100 µg/ml), ß-aminopropionitrile (50 µg/ml) and either 10% delipidized or complete FBS. FBS was delipidized according to the method of Cham and Knowles (20) involving an extraction of the lipid fraction utilizing repeated washing with diisopropyl ether and butanol. Pilot studies indicated that the delipidized FBS was compatible with cell viability for the 96 hour period. Some of the cultures in each treatment group were then further supple-

mented with RET or RA at concentrations of either 10^{-6} or 10^{-7} M. Media containing RET or RA were derived from stock solutions which contained 1.5 mg retinoid/ml 95% ethanol. An equivalent amount of 95% ethanol was added to all control cultures. Cultures were harvested at 48 or 96 hours and the media were assayed for newly synthesized radioactively labeled collagen by a modified pepsin digestion/perchloric acid precipitation procedure (21). Total cell number was determined using a Coulter counter.

RESULTS AND DISCUSSION

In cell cultures which were supplemented with FBS which had been delipidized, a sharp decrease in collagen production was noted when compared with cultures supplemented with complete FBS. At 48 hours, control cultures produced 4,912 $\pm$ 1,029 cpm collagen in the culture medium, whereas cultures supplemented with 10^{-7} M RET or RA produced 15,402 $\pm$ 1,278 cpm and 13,086 $\pm$ 1,177 cpm collagen, respectively ($p < 0.01$ for both RET and RA when compared with controls) (Figure 1). Thus, supplementation with these analogues of vitamin A resulted in approximately a three-fold increase in collagen production. A similar pattern was noted among the various cell cultures at 96 hours, as well (Figure 2). Moreover, differences of a similar

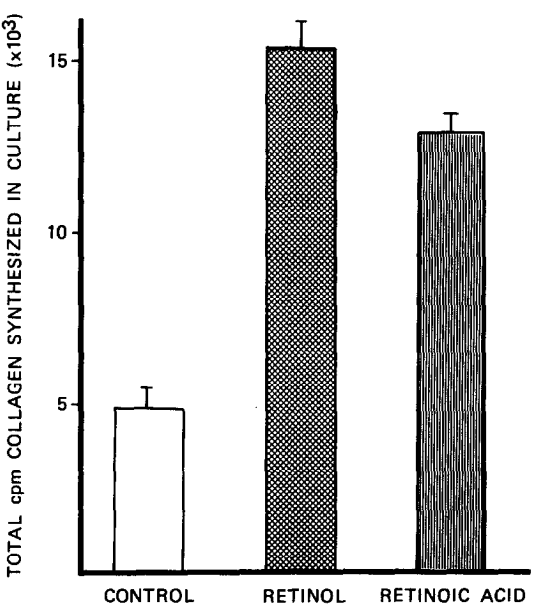


Figure 1: Collagen production by corneal endothelial cells grown in delipidized FBS with and without retinoid supplementation at 48 hours of culture.

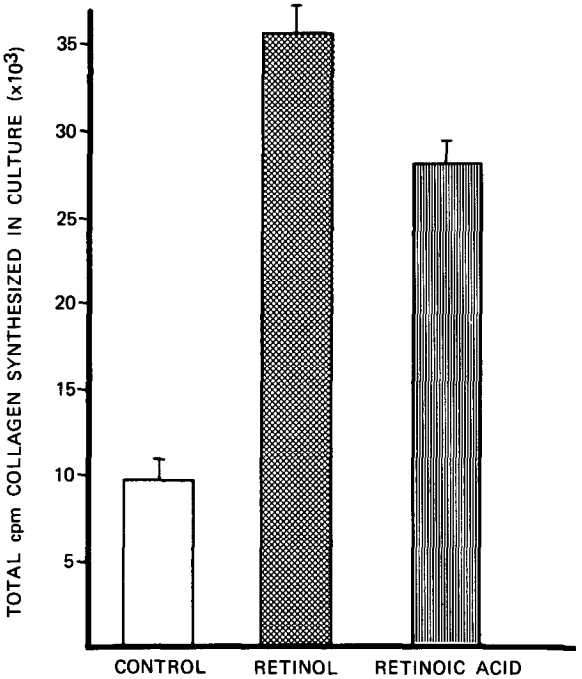
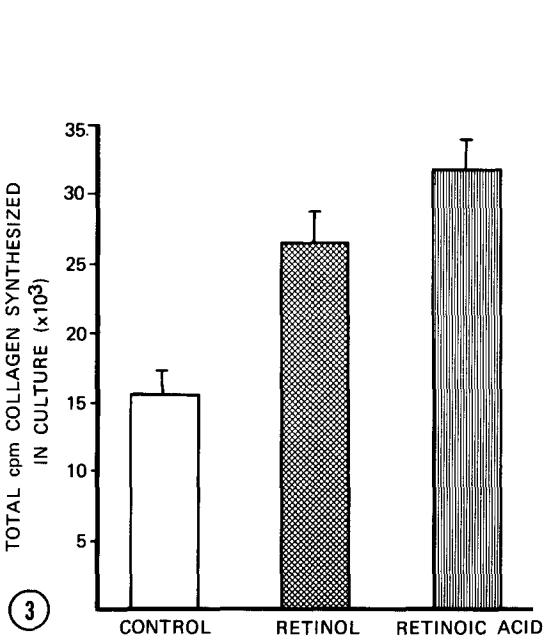


Figure 2: Collagen production by corneal endothelial cells grown in delipidized FBS with and without retinoid supplementation at 96 hours of culture.

magnitude were also noted when collagen production was monitored per individual cell. In addition, total protein production was not altered to the same extent as was collagen production (data not shown). When corneal endothelial cells were cultured in delipidized FBS with 10^{-6} M RET or RA, results were similar to those on which 10^{-7} M RET or RA were employed (results not shown).

When complete FBS (i.e., not delipidized) was utilized and cultures were further supplemented with 10^{-7} M RET or RA, there was an additional nearly two-fold augmentation of collagen production by endothelial cells at both 48 hours (Figure 3) and 96 hours (Figure 4). For instance, at 96 hours, while control cultures supplemented with complete FBS had produced 33,189 $\pm$ 1,921 cpm collagen, those cultures which received additional RET or RA produced 56,072 $\pm$ 2,794 cpm collagen and 62,319 $\pm$ 3,053 cpm total collagen, respectively ($p < 0.01$ for both RET and RA when compared



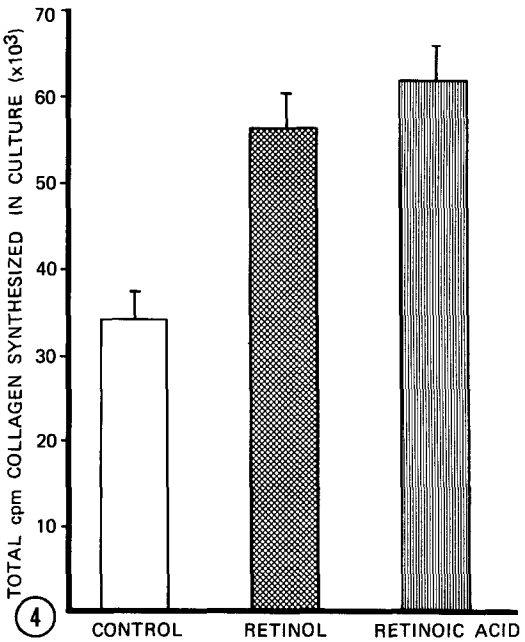


Figure 3: Collagen production by corneal endothelial cells grown in complete FBS with and without retinoid supplementation at 48 hours of culture.

Figure 4: Collagen production by corneal endothelial cells grown in complete FBS with and without retinoid supplementation at 96 hours of culture.

with controls). Again, few notable differences between supplementation with either 10^{-6} or 10^{-7} M RET or RA were observed (results not shown). Therefore, as with the cultures grown in delipidized FBS, both forms of vitamin A, when supplemented at levels higher than those found in complete FBS resulted in a significant increase in collagen production by corneal endothelial cells. Of particular interest, RET was more effective than RA in increasing collagen production when the cells were cultured in delipidized FBS. In contrast, with those cells cultured in complete FBS, RA was more effective in augmenting collagen production than was RET.

The present results have implications for a number of areas of clinical and experimental medicine. Vitamin A deficiency persists as one of the major nutritional deficiencies throughout the world, continuing to blind nearly 1 million children every year (22).

While one of the first signs of vitamin A deficiency is night-blindness, such changes have little to do with the ultimate cause of impaired vision. With a prolonged lack of vitamin A, irreversible degenerative changes in the corneal epithelium and the rapid dissolution of the corneal stroma (stromal "melting") eventually leads to the irreparable loss of vision. Stromal dissolution may occur as a result of exposure of the cornea of the vitamin A deficient organism to aberrantly elevated levels of collagenase, including that produced by infiltrating leukocytes (23). Analogues of vitamin A have been noted to modulate the production of collagenase (24). The inability of various cell types, e.g., stromal fibroblasts and corneal endothelial cells to produce sufficient collagen when vitamin A is not available may also be a critical factor in permitting stromal melting. We suggest that when vitamin A levels in the eye drop below a certain critical value, the balance between synthetic and degradative processes may be lost and collagenous as well as other stromal components may undergo rapid breakdown. The level of collagen production might also be mediated through an influence of vitamin A upon the synthesis of specific glycoproteins such as fibronectin, as we have previously demonstrated (2); the synthesis and cellular accumulation of fibronectin has been shown to be affected by levels of vitamin A (25).

While it is recognized that endothelial cells throughout the body represent a heterogeneous population, the finding that nutrients such as vitamin A are capable of altering phenotypic expression of such cells may have a significance for tissues other than the eye. A prolonged lack of vitamin A, even if of a marginal nature, as might be experienced by significant numbers of the population in developed nations, may lead to the synthesis of defective basement membranes (of which collagen is a major constituent) in the eye and throughout the body. This may lead to an increased incidence of

various degenerative diseases, e.g., increased susceptibility to the establishment of both primary and secondary metastatic tumor foci. The ability of tumor cells to attach to the vessel wall and initiate a tumor focus appears to be dependent upon the presence of specific glycoprotein components (26). Moreover, recent studies have demonstrated that the metastatic potential of different tumor cell lines is correlated with the ability of such transformed cells to degrade the associated basement membrane (27). Thus, a chronic marginal deficiency of vitamin A might lead to an alteration in the presence of specific components as well as the overall structural integrity of the basement membrane, thereby partially accounting for the elevated incidence of a number of types of cancer in individuals with significantly lower levels of serum vitamin A (28-32).

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