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Review

Collagen synthesis in atherosclerosis: too much and not enough

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Abstract

Fibrillar collagen is a critical component of atherosclerotic lesions. Uncontrolled collagen accumulation leads to arterial stenosis, while excessive collagen breakdown combined with inadequate synthesis weakens plaques thereby making them prone to rupture. This review discusses cellular sources of collagen synthesis in atherosclerosis, local and systemic factors modulating collagen gene expression, as well as temporal and spatial patterns of collagen production in human and experimental atherosclerotic lesions.

Keywords: Atherosclerosis; Restenosis; Collagen; Gene expression; Plaque rupture

1. Collagen the good, the bad and the ugly

Collagens are proteins that consist of a triple helix of polypeptide chains and globular domains. Collagens comprise a family of proteins of at least 19 genetically distinct types [1,2]. Human atherosclerotic plaques contain mostly interstitial collagen types I and III [3], and type I collagen comprises approximately two-thirds of the total collagen [4]. Type V collagen also increases in advanced atherosclerotic plaques [5]. Thick type IV collagen depositions are frequently seen in the fibrous cap regions [3,5,6]. This correlates with observation of SMCs surrounded by concentric layers of basement membrane material [7].

Collagen constitutes up to 60% of the total plaque protein [8] thus contributing to the plaque growth and the arterial lumen narrowing. It may also stimulate further lesion progression by serving as a depot for proatherogenic molecules: modified lipoproteins [9], growth factors [10] and glycation end-products [11]. Collagen can modulate macrophage functions [12], SMC proliferation [13], migration [14] and responsiveness to growth factors [15], and stimulate thrombus formation [16]. Fibrillar collagen also contributes to plaque structural integrity and mechanical ''strength''. Therefore, a deficit of collagen reinforcement leads to plaque weakness and vulnerability [17,18]. Thus ''too much'' collagen leads to arterial stenosis, while ''not enough'' collagen makes atherosclerotic plaque prone to rupture.

Collagen content is a net result of dynamic balance between degradation and synthesis. Collagen degradation in atherosclerosis has become a subject of recent reviews [17,19]. This review is focused on collagen synthesis, specifically production of type I and III collagen. Fibrillar collagens are highlighted because they play a major role in both plaque growth and mechanical stability.

2. Principles of collagen biosynthesis

Collagen type I is the product of two different genes, α_1(I) and α_2(I), which are coordinately regulated. Collagen type III is the product of one gene, α_1(III). Transcriptional regulation of type I collagen genes is more thoroughly characterized [20,21]. Regulatory elements located in the promoter and 5′ flanking region and the first and fifth introns of the human and mouse α_1(I) collagen genes have been identified (recently reviewed in [22,23]. Several transcription factors interacting with the proximal

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promoter elements have been identified, including NF-1, SP1, a CCAAT-binding factor, and two inhibitory factors. Regulatory elements of the α_1(I) gene interact with ubiquitous transcription factors that are present in similar amounts in collagen-producing and nonproducing cells. It is suggested that α_1(I) promoter is regulated by cooperative actions of ubiquitous promoter-binding factors and additional factors interacting with other regulatory sites further upstream or downstream, and that the crucial elements and factors have yet to be identified. α_2(I) collagen promoter has a response element at −160 bp that appears to function primarily as a repressor, whereas the other four elements usually function as activators in either coordinate or independent fashion. Transcription factors that interact with α_2(I) collagen promoter include SP1, SP3, AP1 and CBF (reviewed in [23]). Although transcription of the type I collagen gene is activated in human atherosclerotic plaques [24,25], it is unknown if in plaque cells this activation requires a specific ''signature'' of *cis*- and *trans*-elements.

Collagen biosynthesis involves a large number of cotranslational and post-translational events [26]. The intracellular events include formation of pro-α-chains, hydroxylation, glycosylation, assembly and secretion. Extracellular events include cleavage of procollagen molecules, formation of collagen fibrils and cross-linking. Classical steps of collagen biosynthesis have been reviewed in detail [27] and will not be discussed here. Collagen production may be controlled at several levels: (1) transcription, (2) mRNA stability, (3) biosynthesis and activity of each enzyme involved into collagen processing and assembly. Abundant phenomenological data on the regulation of collagen synthesis in atherosclerosis (see Section 3) often fail to pinpoint molecular mechanisms of observed changes.

3. Factors modulating collagen synthesis

Regulation of collagen synthesis is dependent on the intrinsic properties of the cell as well as extrinsic local and systemic factors. Three specific questions will be addressed here: (1) Which cells make collagen in atherosclerotic plaques? (2) Do cell migration and proliferation affect collagen production? (3) How do chemical and physical factors of atherosclerotic milieu influence collagen synthesis?

3.1. Cell types

It is assumed that the bulk of plaque collagen is produced by the smooth muscle cells (SMCs). However, collagens can be also produced by endothelial cells [28]. Because later stage plaques are characterized by capillary vascularization, it is conceivable that some of the synthetic activity is contributed by endothelial cells [24]. It is also known, that not all smooth muscle-like cells in human lesions can be unequivocally identified as SMCs. There is an evidence, that cells different from typical SMCs (for example, stellate intimal cells, osteoblast-like cells, etc.) synthesize type I collagen in human atherosclerotic lesions [29–31]. It is unclear whether these cells represent different cell types or SMC phenotypes (Fig. 1).

3.2. SMC phenotypes, proliferation, migration and collagen synthesis

SMC phenotype, proliferation, migration, and collagen production are central to the pathophysiology of atherosclerosis. However, a functional relationship between them is not well established. Relationships between ''synthetic'' SMC phenotype and collagen synthesis are well elaborated in cell culture, where SMC phenotype varies as a function of cell proliferative state (for review see [32]). In primary cultures of adult rat and rabbit aortic SMCs, the transition into a synthetic phenotype was found to be accompanied by an increase in collagen secretion [33–35]. In subcultured cells, the levels of collagen synthesis were found to show either positive [36,37] or negative [38–40] correlation with SMC proliferation. Transcription factor B-myb represents a potential link in the observed inverse relationships [40].

We correlated cell proliferation and collagen synthesis in human atherosclerotic material [41]. We have demonstrated, that although proliferation and type I collagen gene expression could occur in the same cell, this is a rare event, and the vast majority of collagen-producing cells do not show proliferative activity (Fig. 2).

Collagen synthesis is associated with SMC migration. In

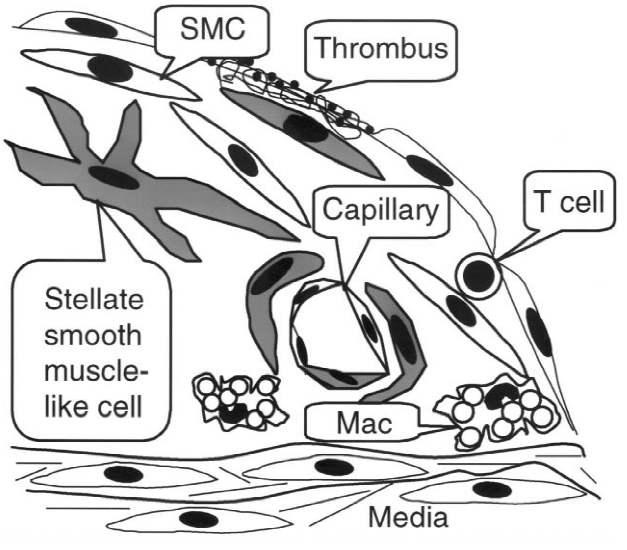

Fig. 1. Type I collagen gene expression in human atherosclerotic plaques (based upon in situ hybridization and immunocytochemical data). Cells with dark cytoplasm represent procollagen-producing cells. SMC, smooth muscle cell; Mac, macrophage.

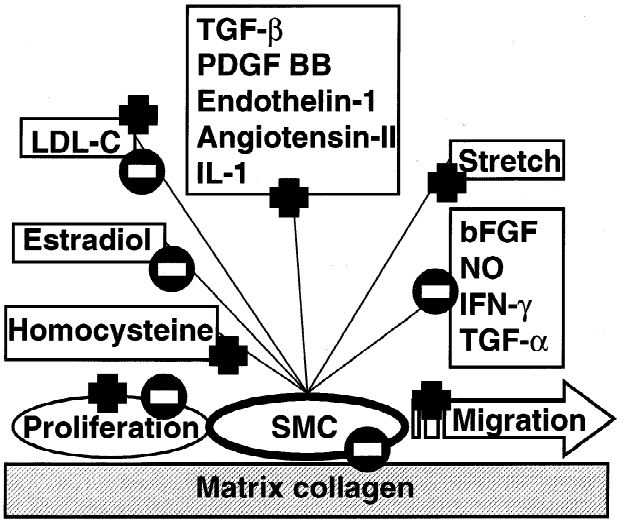

Fig. 2. Factors modulating collagen synthesis. Stimulation and inhibition of collagen production are indicated by + or − signs, respectively. LDL-C, low density lipoprotein cholesterol; TGF, transforming growth factor; IL, interleukin; bFGF, basic fibroblast growth factor; NO, nitric oxide; IFN, interferon; SMC, smooth muscle cell.

vitro inhibition of collagen synthesis affected clustering of β1 integrins on the surface of SMCs, impaired recruitment of vinculin into focal adhesion complexes of spreading SMCs and disassembly of the smooth muscle cytoskeleton, which inhibited SMC spreading and slowed SMC migration [14]. These findings implicate a mechanism whereby newly synthesized collagen may be necessary to maintain the transcellular traction system required for effective locomotion. Thus collagen synthesis facilitates SMC migration. It is unknown though whether cell migration is sufficient for induction or stimulation of collagen production.

3.3. Local factors (Fig. 2)

In SMC culture, transforming growth factor beta (TGF-β) is the most potent and consistent stimulator of collagen synthesis [39,42–44]. TGF-β1 induces overproduction of collagen in a rat balloon injury model [45]. In vivo transfer of TGF-β1 gene into pig artery stimulated type I collagen production and accumulation [46]. TGF-β may elicit its effects both directly and indirectly. A direct mechanism is suggested by the presence of a TGF-β activation element in the promoter of the α_1(I) gene [22]. Indirect effects of TGF-β may be mediated by connective tissue growth factor [47]. TGF-β activity also depends on receptor expression. Normal human SMCs are growth-inhibited by TGF-β, and show little induction of collagen synthesis, yet cells isolated from human atherosclerotic lesions are growth stimulated by TGF-β and markedly increase collagen synthesis [48]. Normal human SMCs express type I, II and III TGF-β1 receptors. The type II receptor is decreased in lesion cells [48]. Genomic instability in the type II TGF-β1 receptor gene has been discovered in human atherosclerotic and restenotic vascular cells [49]. Such receptor-variant cells could overproduce collagen.

Platelet-derived growth factor (PDGF-BB) also stimulates synthesis of type I and III collagen in SMC culture [42]. Transfer of PDGF-B gene stimulates type I collagen production in pig artery [50]. However, some investigators believe that PDGF is not a direct stimulant of collagen synthesis but rather a mitogen for cells, that subsequently synthesize collagen [51]. Interleukin-1 (IL-1) modestly increases the synthesis of collagens I and III [42]. Endothelin-1 [52] and angiotensin-II [53] stimulate collagen synthesis in SMC culture.

In contrast, basic fibroblast growth factor was reported to inhibit both spontaneous [54–57] and TGF-β stimulated [44] collagen production by cultured SMCs. Transforming growth factor alpha also inhibits TGF-β induced collagen synthesis [44]. Both exogenous and endothelial cell-derived nitric oxide inhibit collagen production by cultured SMCs [58,59]. Interferon gamma (IFN-γ), a product of activated T cells, inhibits basal as well as IL-1-, PDGF-, or TGF-β-stimulated collagen synthesis by human SMCs in culture [42]. The antifibrotic effects of IFN-γ have been demonstrated in vivo. When atherosclerosis-prone ApoE-deficient mice were crossed with IFN-γ receptor-knock-out mice, the mice exhibited a reduction in atherosclerotic lesion size, but a marked increase in lesion collagen content [60]. IFN-γ also has been shown to inhibit intimal thickening in the rat carotid balloon injury model [61,62].

Extracellular matrix itself controls collagen synthesis in SMC culture. SMCs produced less collagen on fibronectin-coated surfaces compared with cells grown on albumin-coated dishes [63]. Cells seeded on top or within a collagen gel showed a suppressed collagen synthesis compared with cells grown directly on plastic [64]; this suggests an autoregulatory mechanism. The steady-state levels of α_1(I) and α_1(III) collagen mRNA of cells within collagen lattices were higher that those grown on plastic, although the production of collagen was lower [65]. These data suggest the involvement of post-translational control of collagen production in collagen lattice-cultured SMCs. It has been also demonstrated, that preexisting collagen matrix may modulate growth factor-induced collagen synthesis.

Mechanical stretch can stimulate collagen synthesis by cultured SMCs [66–68] or whole artery segments [69,70] and may thus be a link between local hemodynamic forces and plaque collagen production. The stretch-induced collagen synthesis appears to be mediated via an autocrine–paracrine mechanism of angiotensin II and TGF-β released from SMCs [71].

3.4. Systemic factors (Fig. 2)

Ironically, the level of circulating low density lipop-

rotein-associated cholesterol (LDL-C) is widely used as a predictor of both plaque growth [72] and rupture [73], i.e. the situations characterized by allegedly excessive and insufficient collagen synthesis, respectively. Attempts to define the direct effects of LDL-C on collagen production by cultured SMCs rendered contradictory results. Oxidized human LDL stimulates collagen production in cultured porcine [74] and rabbit [75] SMCs. Incubation of cells isolated from human aortic intima with sera of atherosclerotic patients, but not of healthy donors, enhanced collagen synthesis [76]. The LDL fraction had the same effect as whole serum. On the contrary, serum from type IIA hypercholesterolemic patients inhibited collagen production by cultured human fetal aortic SMCs [77]. Similar results were demonstrated in analogous rabbit [78] and monkey [79] systems. Variable sources of cultured cells and techniques of LDL or serum handling make comparison of these data difficult. Moreover, in vivo LDL-C is mostly accumulated by macrophages, which drastically changes macrophage biology. Products of lipid-laden macrophages may influence ability of adjacent SMCs to synthesize collagen. Thus, in vivo influences of LDL-C on collagen synthesis represent the result of complex direct and indirect interactions with SMCs and therefore not necessarily can be predicted by in vitro incubation of SMCs with LDL-C.

Homocysteine is also a risk factor for atherosclerosis [80]. Cultured SMC treated with homocysteine at concentrations observed in patients with hyperhomocysteinemia had collagen synthesis rates as high as 214% of control values [81].

At the same time, antiatherogenic effects of some systemic factors can be mediated by inhibition of collagen synthesis. Administration of an estrogen–progesteron combination to intact female rabbits on an atherogenic diet inhibited collagen synthesis and retarded the development of atherosclerosis [82]. Ovariectomy increased the synthesis of aortic collagen and development of atherosclerosis in rabbits on atherogenic diet, whereas the administration of estradiol to similarly manipulated rabbits inhibited this increase [83]

4. Time course of collagen synthesis in atherosclerosis

4.1. Human atherosclerosis

Neither we nor others have found any type I collagen producing cells in normal human arteries [24,31,84,85]. The presence of type I procollagen cells has been recently reported in all types of human aortic lesions: ~6% in initial lesions, ~18% in fatty streaks and fibrolipid plaques and ~7% in fibrous plaques [31]. Thus collagen synthesis occurs very early in lesion development and may represent the major mechanism of its progression.

4.2. Animal models

We failed to find any formal time course study in a rabbit hypercholesterolemic model. It can be inferred from different publications, that increased collagen synthesis was detected at 2 [86], 3 [82,87], 4 [88] and 6 months [89] after initiation of cholesterol feeding. In fact, 2–3 month old rabbit lesions are considered analogous to human fatty streaks.

In the rat carotid artery balloon injury model, after 2 weeks, while cell proliferative activity is returning to control levels, the intima continues to enlarge [90]. Type I procollagen mRNA levels showed an initial decrease at 2 days, significantly increased at 1 week, peaked at 2 weeks, then diminished at 4 weeks after injury [91]. Cultured neointimal SMCs, obtained from rabbit aortas, exhibited elevated type I and III collagen gene expression 15 weeks after balloon injury [92]. Thus during the short-lived proliferative phase and predominantly thereafter much intimal enlargement is a result of extracellular matrix expansion largely due to collagen.

5. Spatial patterns of collagen synthesis (Fig. 1)

5.1. Plaque topography and collagen synthesis

Type I and III collagen synthesis tends to be located in the intima [24,25,31,84,85,93]. We found that in advanced fibrous carotid and coronary plaques, type I procollagen-synthesizing cells were especially prevalent in fibrous cap and vascularized parts of the shoulder regions [24]. The reason for clustering of collagen-producing cells is unknown. Most likely, it reflects the concentration gradient of various regulatory factors, although selection of ‘‘fibrogenic’’ cells is also a possibility.

5.2. Macrophages

A putative inflammatory link to collagen gene expression in human atherosclerosis has been highlighted by Jaeger et al. [25]. They found higher collagen type I and III mRNAs in human intimas than in medias. The authors suggested that expression was in SMCs adjacent to macrophages. Liptay et al. have demonstrated colocalization between type I collagen gene expressing SMCs and nonfoamy neointimal macrophages [93]. An association between type I collagen gene expression and monocyte/macrophages in human hypertensive pulmonary arteries has also been demonstrated [94]. We also have detected higher type I collagen mRNA and type I procollagen protein expression in human carotid and coronary atherosclerotic plaques that in normal coronary or internal mammary arteries, but have not seen a spacial correlation with the presence of macrophages [24]. Jaeger et al. reported no collagen/macrophage association in human aortic coarcta-

tions [95]. In addition, the rat carotid artery injury model does not exhibit significant numbers of macrophages, but it does show prominent collagen gene expression. Thus, macrophages may be involved in, but are not necessary for collagen synthesis.

5.3. T cells

Our morphometric analysis revealed strong negative association between plaque regions displaying type I collagen gene expression and the presence of T cells, suggesting that T cell mediators such as IFN-γ [42] inhibit collagen synthesis in human atherosclerosis.

5.4. Plaque microvessels

We have also demonstrated that much of type I collagen synthesis takes place in the vicinity of plaque microvessels [24,30]. Both endothelial cells and surrounding SMCs (pericytes?) synthesized collagen. This might be because capillaries deliver serum-derived growth factors or because neovascularization is associated with production of growth factors. On the other hand, type I collagen may be important factor controlling plaque neoangiogenesis. Type I collagen induces endothelial cells to form capillary tubes in vitro [96]. It was proposed, that collagen fibrils serve as a template or cable onto which endothelial cells wrap themselves [97].

5.5. Thrombus

Our studies of human atherectomy coronary samples revealed close association between type I collagen gene expression and mural thrombi [30] in both primary and restenotic lesions. Exact reasons for such association are unclear. Thrombus may directly release growth factors and/or its fibrin constituent may work as a scaffold for migrating SMCs with a secondary activation of collagen synthesis. In general, colocalization of collagen synthesizing cells and thrombotic material may reflect a process of mural thrombus organization as a part of wound healing after asymptomatic plaque rupture thus providing ''growth through plaque rupture'' [17].

5.6. Implications for plaque growth and rupture

Collagen degradation and synthesis take place simultaneously within the same plaques [85]. We are not aware of any study where both processes have been topographically correlated. However, overlaying the collagen synthesis data with the data on localization of collagen-degrading activities could be instructive. Matrix metalloproteinases (enzymes responsible for collagen degradation) are most often located in foamy macrophages within plaque shoulders and are rarely seen within the fibrous cap (reviewed in [17]). It can be therefore implied that fibrous cap area would have a tendency to grow. The fate of a shoulder region is unpredictable, since two conflicting processes (degradation and synthesis) are colocalized. However, with any given rate of degradation, the less the rate of collagen synthesis, the more vulnerable the plaque. There are two major potentials for inadequate collagen synthesis in the plaque shoulders: (1) high concentration of the inhibitors of collagen synthesis, like T-cell derived IFN-γ; (2) local depletion of the cellular source of collagen synthesis, i.e. SMC death. Both processes do in fact take place in human atherosclerotic plaques [98,99].

We have recently developed an animal model of atherosclerosis, where the plaque is formed around inflatable balloon and can be ruptured at will [100]. We have demonstrated that hypercholesterolemia (known predictor of plaque destabilization) impaired the lesion's mechanical properties. In hypercholesterolemic rabbits, the number of type I procollagen-synthesizing cells was increased in both fibrous cap and shoulder regions of the plaque. However, fibrous caps were collagen-rich, while shoulders were collagen-poor. At the same time, fibrous caps contained numerous SMCs and almost no macrophages, while shoulders contained very limited numbers of SMCs and plenty of macrophages (Fig. 3). As a result, collagen degradation prevailed in plaque shoulders and led to plaque mechanical weakening [in preparation]. These results show that in our model (1) hypercholesterolemia simultaneously stimulated plaque growth and destabilization, (2) destabilization was determined by the loss of cellular source rather than inhibition of collagen gene expression.

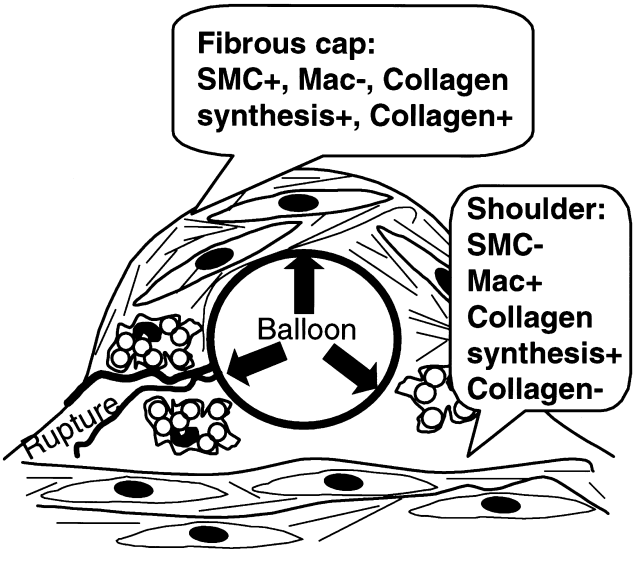

Fig. 3. Novel rabbit model of atherosclerotic plaque rupture. A plaque can be ruptured at will after an inflatable balloon becomes embedded into the plaque. Rupture occurs in collagen-depleted regions. SMC, smooth muscle cell; Mac, macrophage.

6. Collagen synthesis in restenosis after angioplasty

Collagen fibers occupy about 80% of the section area in the samples of human coronary restenotic lesions [101]. Nikkari et al. described elevated type I collagen synthesis in human carotid restenosis [85]. We were unable, however, to find any dramatic difference in type I procollagen positive cell number in human primary and restenotic lesions [30].

Reinjury of rat arterial lesions induced an increase in lesion size that was not associated with an increase in cell numbers [102]. Type I collagen gene expression was elevated 7 days after reinjury and returned to the control levels by 28 days. In rabbit double-injury model collagen synthesis was increased up to 4–10 times above control, as noted at 1, 2, and 4 weeks after angioplasty. The increase in synthesis was accompanied by a significant increase in collagen content that coincided with the increase in cross-sectional area [103]. Collagen synthesis and degradation showed similar temporal profiles. Peak collagen synthesis and degradation occurred at 1 week after angioplasty. Interestingly, MMP inhibitor reduced both collagen degradation and synthesis [104]. These data suggest that degradation of newly synthesized collagen is an important mechanism regulating collagen accumulation and that MMPs have an integral role in collagen turnover after balloon angioplasty.

Increased synthesis, however, does not always mean collagen accumulation and the luminal narrowing. Coats et al. reported that collagen content was significantly lower in rabbit restenotic vessels [105]. Geary et al. argue that lumen narrowing is caused in large part by changes in artery wall geometry rather than intimal mass per se [106]. They suggest that newly synthesized collagen may work as a substrate for integrin-dependent wound contraction leading to luminal narrowing.

7. Is collagen synthesis a reasonable target for anti-atherosclerotic therapy?

7.1. Inhibition?

Traditionally, atherosclerosis was equated with luminal narrowing as a result of intimal growth. From that standpoint, any treatment inhibiting intimal thickening via inhibition of collagen synthesis should have beneficial effects. The list of compounds demonstrating ability to inhibit collagen synthesis by SMCs, includes, but is not limited to Ca^{2+} channel blockers [107], nitric oxide generators [58], derivatized dextrans [108], tranilast [109], protamine [110], halofuginone [111], and L-mimosine [112]. However, inhibition of collagen production may shift the balance toward matrix breakdown thus making plaques prone to rupture. Additional studies are necessary to address this still-theoretical concern.

7.2. Stimulation?

Conceptually, stimulation of collagen production in atherosclerotic patients may be counterintuitive. If administered long term, such therapy might stabilize some plaques, but accelerate an overall progression of atherosclerosis and induce fibrosis of various organs. However, some compounds can stimulate collagen production, yet overall have an anti-atherosclerotic effect. For example, tamoxifen elevates TGF-β and suppresses diet-induced formation of lipid lesions in mouse aorta [113]. Although collagen synthesis was not evaluated in that report, the general knowledge of TGF-β biology predicts stimulation of collagen production.

In rabbits, lipid lowering by diet is sufficient to increase collagen content in atheroma via reduction of matrix metalloproteinase-driven collagen degradation [114]. Therefore, long term successful lipid lowering can stabilize plaques without pharmacological stimulation of collagen synthesis. However, there may be some specific short-term goals for boosting collagen production. Increase of collagen breakdown was demonstrated in patients with myocardial infarction treated with streptokinase or tissue plasminogen activator [115,116]. This life-saving intervention may increase the risk of plaque rupture. Stimulation of collagen synthesis may help to counterbalance matrix breakdown.

Progress in clinical imaging makes identification of ruptured or vulnerable plaques feasible in the nearest future [117]. Once the culprit plaque is identified, it may be selectively treated by means of gene therapy. We have demonstrated in organ culture experiments, that areas of plaque rupture and thrombus are sites of predilection for expression of recombinant genes, since these areas are devoid of collagen, and, therefore, do not possess an anatomical barrier for vector penetration [118]. Overexpression of genes stimulating collagen production has a potential to increase plaque strength.

8. Summary

Uncontrolled collagen accumulation leads to arterial stenosis, while excessive collagen breakdown combined with inadequate synthesis weakens plaques thereby making them prone to rupture. Further studies focused on molecular regulation of collagen synthesis and degradation are necessary to better understand the mechanisms of development and complications of atherosclerosis.

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