INTRODUCTION

Gingival fibromatosis (GF) comprises a rare clinical condition characterized by abnormally diffuse or localized growth of the maxillary and mandibular gingiva. The condition among others occurs as a side effect of some medications. It may be related to hereditary factors or in some cases the aetiology of the enlargement remains unknown (1-3). Hereditary gingival fibromatosis (HGF) may manifest as an autosomal-dominant or, less commonly, an autosomal-recessive mode of inheritance, either as an isolated disorder or as part of a syndrome (2, 4-6). Autosomal dominant forms of GF, which are usually non-syndromic, have been genetically linked to the chromosomes 2p21-p22 and 5q13-q22. Recently, a mutation in the \textit{Son-of-Sevenless} (\textit{SOS-1}) gene has been suggested as a possible cause of isolated (non-syndromic) gingival fibromatosis. \textit{SOS-1} is a guanine nucleotide-exchange factor that functions in the transduction of signals that control cell growth and differentiation. A single base insertion mutation (\textit{SOS-1} g.126,142-126,143insC) introduces a frameshift, creating a premature stop codon, abolishing 4 functionally important proline-rich SH3 binding domains normally present in the carboxyl-terminal region of the \textit{SOS-1} protein (7). The resultant protein chimera has enhanced activity, since it lacks the carboxyl-terminal domain that normally exerts negative allosteric control (8). This mutation causes HGF in humans, and similar \textit{SOS-1} deletion constructs are functional in animal models, and a transgenic mouse construct with a comparable \textit{SOS-1} chimera produces a skin hypertrophy phenotype (7, 9-11). A mutation in \textit{SOS-1} gene has been associated with increased fibroblast proliferation and collagen matrix synthesis in HGF (12).

Although the overgrown tissue does not cause a pain, it may eventually cover most of the teeth. The condition may hinder oral cavity hygiene and result in underlying bone loss. The long-term benefits of surgery cannot be predicted. On the other hand, alternative, efficient and non-invasive methods are not available at present. The aim of this study was to test the inhibitory effects of a chimeric IgG variant on collagen fibril formation in the cell culture of gingival fibroblasts taken from a patient with hereditary gingival fibromatosis with a high propensity for recurrence. Gingival biopsies were collected from the mandibular gingiva and used for histological evaluation as well as to establish a fibroblast culture. A histological evaluation was made in haematoxylin-eosin and Heidenhain's trichrome stained tissue sections. The inhibitory effect of a chimeric antibody on collagen fibril formation was determined in fibroblast cultures by using a collagen-specific Western blot and immunofluorescent staining. A histological evaluation revealed epithelial acanthosis with singular elongated rete pegs extending into the underlying connective tissue stroma that consisted of locally abundant, irregular collagen bundles. Based on observations with an \textit{in vitro} model we conclude that a chimeric anti-collagen antibody efficiently inhibits collagen fibril accumulation in cell culture derived from diffuse, hereditary gingival fibromatosis that is characterized by a high propensity for recurrence (high proliferation index). Employing cell cultures from standardized group of patients with recurrent hereditary gingival fibromatosis as well as standardizing relevant 3D (tissue-like) models will be crucial for further tests of the antibody.

\textbf{Key words:} hereditary gingival fibromatosis, recurrence, collagen fibrils, chimeric antibody
elucidated (19-22). The most attractive concept is that molecular matrix remodeling programs are initiated in epithelial cells that enable these new motile and transitioning cells to invade through basement membranes and migrate into connective tissue stroma where they contribute to new extracellular matrix (ECM) production (21). Epithelial-to-mesenchymal transition (EMT) is a process in which epithelial cell-cell and cell-extracellular matrix interactions are weakened as epithelial cells trans-differentiate into fibrogenic fibroblast-like cells. Although a normal process in development, EMT underlies pathological processes in mature tissues including progression of tumors of epithelial origin, and heart, lung, liver, and kidney fibrosis (20). Additionally, in this process are involved several pro-fibrogenic cytokines and receptors, such as connective tissue growth factor (CTGF) and transforming growth factor β (TGFβ) which are considered the main inducers of EMT (23, 24). GF is characterized by elevated levels of pro-fibrogenic cytokines, including TGFβ and CTGF. Thus, another concept is that growth factors trigger fibrosis via direct regulation of molecules involved in collagen and other ECM components synthesis. At present, appropriate oral hygiene and scaling are sufficient for localized and minimal enlargements, while cases of massive gingival overgrowth require surgical correction (25). On the other hand, the inaccessibility of alternative, effective, and less invasive methods indicates on the importance of discerning studies testing novel tools that might become valuable to GF treatment in the future.

Recently, it has been proposed that the extracellular process of collagen fibril formation may serve as a valid target for reducing collagen deposits in fibrotic tissues. It has been demonstrated that employing a monoclonal antibody that targets the C-terminal telopeptide of the α2 chain of collagen I blocks critical collagen I-collagen I interaction, thereby reducing the amount of collagen deposits in vitro and in models representing growth of fibrotic tissues (26). Furthermore, recently Fertala et al. shown efficient inhibition of collagen fibril formation in vivo and in cultures of various cells associated with the fibrotic process that develops after trauma by using a novel chimeric antibody variant (chIgG) (27).

The objective of this study was testing in in vitro conditions the inhibitory potential of a novel chimeric chIgG in a highly recurrent diffuse hereditary gingival fibromatosis.

MATERIAL AND METHODS

The study was carried out in accordance with the Declaration of Helsinki and the consent of the patient and healthy subjects was obtained prior to the study as approved by the Bioethical Committee of the Jagiellonian University, Medical College in Cracow, Poland.

Histology of gingival biopsies from hereditary gingival fibromatosis

Two gingival biopsies (1 × 2 mm, 2 × 2 mm) obtained from the anterior region of the mandibular gingiva of HGF patient were fixed in 10% formalin in a phosphate buffered saline (PBS) for 24 hours at 4°C. The tissue was dehydrated, paraffin embedded and serially sectioned (thickness 7 µm). A histo-morphological evaluation was conducted in gingival sections stained according to the haematoxylin-eosin method and using a fluorescent microscope (Nikon Eclipse Ti, Japan) connected to NIS-Elements F 3.0 software (Nikon Inc.). Heidenhain's trichrome stain was used to visualize collagen fibers in tissue sections.

Cell culture

Gingival biopsies from patient with hereditary gingival fibromatosis and from controls were used to establish fibroblast cultures. In brief, following the enzymatic separation of the epithelial layer the remaining connective tissue was digested with 0.1% collagenase I (Invitrogen). After digestion, the cell pellet was plated in T25 flask in Dulbecco's Modified Eagle Medium (DMEM, PAA GmbH) supplemented with 10% heat-inactivated foetal bovine serum (FBS, PAA), penicillin/streptomycin (50 µ/l), gentamicin (50 µ/l), and nystatin (10 µg/ml, PAA), at 37°C in a humidified atmosphere containing 5% CO₂. The homogeneity of the gingival fibroblast culture was confirmed after passage 1 by immunofluorescent staining (vimentin+, cytokeratin–). Gingival fibroblast cultures at passage 3–5 were used for the experiments. Cell viability was confirmed by Trypan blue staining and amounted to 97–99%.

Inhibition of collagen deposition formation in fibroblast cultures in the presence of the anti-α2CII-αIgG.

Western blot

In brief, for Western blot assays, 50 × 10³ cells per well were seeded into a 24-well plate. Cell cultures were overlayed with a fresh medium supplemented with 40 µg/ml of L-ascorbic acid phosphate magnesium salt n-hydrate (Wako Pure Chemical Co.) and a mixture of 70-kDa and 400-kDa Ficol (Sigma-Aldrich) added at concentrations of 40 mg/ml and 25 mg/ml, respectively. Subsequently, αIgG was added to the medium at a concentration of 70 µg/ml. Cell cultures were maintained for 6 days and the medium was changed every 2 days. The optimal concentration of αIgG for cell culture utility and the cytotoxic assays were standardized previously (27). Cell lysates were collected by employing a lysis buffer consisting of 1% SDS, 1% sodium deoxycholate, 0.1% Triton X-100, 10 mM EDTA, 3% β-mercaptoethanol and a mixture of protease inhibitors (Thermo Scientific). The lysates (60 µg of protein, a Bradford protein assay) were electrophoresed in polyacrylamide gels followed by their transfer onto nitrocellulose membranes. Collagen I was detected using a monoclonal antibody (DAKO), and using the optical density of bands on the immunoblot for quantitative purposes. The histology of gingival biopsies from hereditary gingival fibromatosis.

Immunofluorescent staining

For the immunostaining assays, 15 × 10³ cells per well were seeded into an 8-chamber slide. Further procedures were performed identical to the Western blot assays. The medium was supplemented with αIgG (70 µg/ml). Cell cultures were maintained for 6 days and the medium was changed every 2 days. The cells were fixed with ice-cold methanol, rinsed with...
PBS and then collagen fibrils were immunolocalized with the primary polyclonal rabbit anti-collagen I antibodies, followed by the secondary anti-rabbit IgG antibodies conjugated to Alexa Fluor 594 (Invitrogen). The nuclei of the examined cells were stained with 4',6-diamidino-2-phenylindole (DAPI). The immunofluorescent reaction was analyzed with a fluorescent microscope (Eclipse E600, Nikon Inc.). Microphotographs were obtained with a digital camera (DS-Qi1Mc, Nikon Inc.) controlled by the NIS-Elements software (Nikon Inc.).

RESULTS

Histological evaluation of gingival biopsies from patient with hereditary gingival fibromatosis

A microscopic evaluation revealed epithelial acanthosis with singular elongated or enlarged rete pegs extending into underlying connective tissue stroma (Fig. 1A-1C). Locally abundant, irregular collagen bundles were visualized within the connective stroma using Heidenhain's trichrome stain (Fig. 1D-1F). Inflammatory cells were distributed throughout almost the entire connective tissue (Fig. 1A-1C). Inflammatory infiltrates were characterized by the prevalence of oval-shaped cells with a round, eccentrically located nucleus and an abundant basophilic cytoplasm. The nuclear chromatin of those cells was coarse and distributed in a pattern sometimes resembling the spokes of a wheel (cartwheel nucleus). The other predominant cell population found was represented by large mononuclear cells with a vesicular, elongated or kidney-shaped nucleus with a clearly visible nuclear membrane as well as an abundant, and eosinophilic cytoplasm. Two predominant populations of cells that infiltrated the overgrown gingival tissue were identified as plasmocytes and histiocytes, respectively. The third and less numerous population was represented by lymphocytes.

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**Fig. 1.** Histological presence of hereditary gingival fibromatosis: epithelium and lamina propria visible in sections stained with haematoxylin and eosin, original magnification 100 × (A–C); collagen fibrils stained with Heidenhain’s trichrome, magnification 100 × (D–F). Bars = 100 µm.

**Fig. 2.** Western blot assays of collagen I extracted from primary gingival fibroblast cultures derived from a patient with recurrent, diffuse, hereditary gingival fibromatosis, A; and from controls, B. Cells were cultured with the inhibitory α1IgG variant of the anti-α2Ct antibody (A - left; B - middle); cells cultured with α1IgG (A - right; B - right) or in the absence of IgG (A - middle; B - left). Experiments were repeated three times and representative images of Western blots are presented. Symbols: inhibitory chimeric IgG, inhibitory α1IgG variant; human non-inhibitory IgG, α1IgG; No IgG, sample not treated with any IgG variant; Collagen I, partially processed pro-α1 chains and cross-linked forms of α1 chains; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; indicates uniform gel loading and provides a reference for the α1 collagen chain.
The inhibitory effect of chimeric anti-α2Ct IgG on collagen fibril formation

Collagen fibril inhibition was measured using Western blot and immunostaining assays. In the case of Western blot we used cell lysates from fibroblast cultures maintained for 6 days in the presence of therapeutic α1IgG at a concentration of 70 µg/ml. As shown in Fig. 2, α1IgG almost completely blocked collagen fibril formation, while the same effect was not observed after cells were treated with a non-inhibitory α1IgG or in the absence of IgG. Microscopic analysis revealed relatively abundant collagen I fibers in the presence of control α1IgG (Fig. 3A). After treatment with therapeutic α1IgG fibril deposits had been reduced to almost zero (Fig. 3B). Gingival fibroblast cultures established from healthy subjects were used as controls (Fig. 3C-3D).

DISCUSSION

GF is a condition characterized by progressive enlargement of the gingiva. A common feature of the condition is a problem with maintaining oral hygiene, speech disturbances, and difficulties with mastication (13, 14, 28). If left untreated, the enlarged tissue may eventually cover most of the teeth and because of the difficulties in maintaining oral hygiene, cause bone loss. The condition may also induce caries development and other inflammatory periodontal disorders (11, 13, 23). Additionally, oral infections stemming from gingival overgrowth in patients with unbalanced normal microbiota of oral cavity may lead to activation of Th17 cells and macrophages which upon induction produce excessive levels of proinflammatory cytokines. This process, in turn, can potentially impair systemic health of the patients (29, 30). Epithelial acanthosis with its potential cosmetic implications and tendency to provide a niche for further growth of microorganism, the build-up of plaque, and gingival inflammation, is still challenge for periodontists to diagnose and manage effectively.

Hereditary gingival fibromatosis may be transmitted as a Mendelian trait, and both autosomal dominant and autosomal recessive transmission have been reported (31). Linkage studies have localized loci for isolated nonsyndromic autosomal dominant forms of gingival fibromatosis to chromosome 2p21-p22 (32) and to chromosome 5q13-q22 (33). In addition to nonsyndromic forms, GF has been associated with a variety of syndromic manifestations. Hart et al. performed sequence analysis of 6 known candidate genes, as well as 10 additional, previously uncharacterized genes within the refined interval, and identified a mutation in the SOS-1 gene, that segregated with the HGF phenotype in a large group of members of a Brazilian family (7). It has been suggested that the g.126,142-126,143insC mutation produces the truncated protein chimera p.K1084fsX1105 that causes HGF through a gain-of-function mechanism. However, why the HGF phenotype appears localized to the gingiva is unknown. The keratinized masticatory mucosa is recognized to be developmentally unique, and different tissue-specific signaling pathways in this unique tissue may be responsible for the limited tissue distribution of the GF phenotype. Although the sites-specific localization of this phenotype is remarkable, the gingiva is also the only site affected in drug-induced and most syndromic forms of GF. The SOS-1 mutation in individuals with HGF described by Hart et al. identified the first genetic mutation for isolated HGF (7). Identification of the specific genetic basis in HGF should help to elucidate the pathogenic mechanism by which gingival overgrowth occurs (34). Otherwise, hereditary gingival fibromatosis displays genetic heterogeneity, and mutations in other genes are likely involved (35, 36). In turn, Lee et al. (12) analyzed histological, morphological, and proliferation characteristics in monolayer and three-dimensional cultures of fibroblasts with the SOS-1 g.126,142-126,143insC mutation. Histological assessment of HGF gingiva indicated increased numbers of fibroblasts (30%) and increased collagen (10%). Cell proliferation studies demonstrated increased growth rates and 5-
bromo-2-deoxyuridine incorporation for HGF fibroblasts. Flow cytometry showed greater proportions of HGF fibroblasts in the G2/M phase. Subsequently, HGF derived fibroblasts in three-dimensional culture showed greater cell proliferation, higher cell density, and alteration of surrounding collagen matrix. The observations from this study clearly shown that increased fibroblast numbers and collagen matrix changes are associated with mutation of the SOS-1 gene in vitro and in vivo (12). Increased fibroblasts proliferation and extracellular matrix deposition in HGF was also reported by others (16, 17, 37-40).

Recently, it has been proposed that the extracellular process of collagen fibril formation may serve as a valid target for reducing the mass of collagen-rich fibrotic tissues. It has been postulated that targeting the specific process of fibril formation may offer not only an effective but also a safe way to reduce collagen synthesis in fibrotic tissues. Since fibril formation is driven by telopeptide mediated collagen-collagen interaction, an anti-α2Ct antibody was generated that specifically binds to the telopeptide of the α2 chain of human collagen I, thereby blocking its critical binding interaction (26, 41). Consequently, the anti-α2Ct antibody was tested as the inhibitor of localized fibrosis in a keloid-like model and these initial tests indicated the great potential of the anti-α2Ct antibody to reduce the mass of the formed fibrotic tissue (26). However, the prototypic anti-α2Ct antibody originates from mice and it belongs to the IgA class. As a consequence, its utility as a therapeutic agent in human patients is limited. Subsequently, the same group engineered a chimeric antibody variant (αIgG), consisting of variable domains derived from the prototypic IgA-type anti-α2Ct antibody and the constant domains of the human IgG (IgG). The novel antibody was tested for its ability to inhibit collagen fibril formation in vitro and in cultures of various cells associated with the fibrotic process that develops in response to trauma (27). According to above evidences we proposed herein to study a novel approach to treating gingival overgrowth lesions using a novel chimeric IgG. To test the inhibitory potential of a novel anti-collagen IgG we used gingival fibroblasts from a patient diagnosed with diffuse, hereditary non-syndromic gingival fibromatosis with the recurrence of about 12 months after complete excision of the lesion. Histological evaluation of gingival sections revealed epithelial acanthosis with singular elongated or enlarged rete pegs extending into the underlying connective tissue stroma. Abundant, irregular collagen bundles were found locally in the stromal part of the gingiva. We observed an approximately 2.7-fold increase in the expression of procollagen I and a roughly 2-fold increase in proliferation at 72 hours in fibroblast cultures derived from this patient as compared to controls (data not shown). The tests conducted in the present study shown almost complete inhibition of collagen fibril formation, and this effect was not detected in cells supplemented with non-inhibitory IgG or in the absence of IgG (Fig. 2). This observation was confirmed by immunofluorescent microscopic analysis of cell cultures treated with non-inhibitory IgG (Fig. 3A), or therapeutic αIgG, respectively (Fig. 3B).

As shown by others a mutation in SOS-1 gene has been associated with increased fibroblasts proliferation and collagen matrix synthesis in HGF (12). Increased proliferation was also noted in fibroblasts used in the current study that were isolated from hereditary gingival overgrowth (unpublished data). Therefore another attempt to counteract gingival overgrowth is reducing the mass of collagen-rich fibrotic tissues. It has been shown that treatment of DPSCs and HDFs with clinical concentrations of MTX resulted in stress-induced premature senescence (SIPS) that is a permanent cell cycle arrest in response to DSBs observed in some cell types. It was also found that lower concentration of MTX induced SIPS, while higher concentration of MTX induced programmed cell death, which was accompanied by increased expression of proapoptotic protein puma and activation of effector caspases 3/7 in both cell types. The results presented by Seifrtova et al. are interesting and such approach would seem promising also with respect to counteract gingival overgrowth. However, first, relevant studies are needed that would clarify the effect of MTX on gingival fibroblasts derived from HGF lesions, cells susceptibility and response to this drug as well as its mechanisms of action.

Another interesting approach concerns employment of bone marrow mesenchymal stem cells (BMSCs) and/or mesenchymal stem cells (MSCs) of oral origin to therapy of a number of periodontal diseases. DPSCs and periodontal ligament stem cells (PDLS) are of particular interest. An abundant source of both cell types are teeth (45) and MSCs are normally obtained from teeth removed during routine dental care. It has been shown that DPSCs and PDLSCs induced apoptosis of some cells in vitro conditions (47, 48). Moreover, transplanted BMSCs in response to appropriate pharmacological induction differentiated into cementoblasts, osteoblasts, osteocytes and fibroblasts of the regenerated tissue. Therefore replacement of abnormal fibroblasts from overgrown gingival tissue by allogeneic and/or autologous drafts of fibroblasts of MSCs origin, by DPSCs or PDLSCs would be of some importance in supporting therapy (regenerative phase) of gingival overgrowth lesions. Although MSCs from dental sources such as the dental pulp, the periodontal ligament and the gingiva represent a high potential for tissue regeneration, its biology and “behaviour” within regenerating tissues is yet not fully explored, indicating the need for more research to speed the path to clinical applications.

Histological evaluation of gingival sections in the current study revealed infiltrates of immune cells throughout almost the entire connective tissue i.e. subepithelial connective tissue, around vessels and capillaries, and within the connective stroma. Epithelial acanthosis that lead to partial or complete coverage of teeth provides a niche for microorganisms growth and the build-up of plaque and gingival inflammation. If left untreated such a condition may induce inflammatory diseases of the periodontium. The risk of gingival inflammation is higher in patients with unbalanced composition of normal oral microbiota, e.g. lower amount of Lactobacilli spp. and decreased production of H₂O₂ that negatively correlates with activation of Th17 cells, and increased levels of IL-17 as well as TNFα (29). Activation of Th17 cells and macrophages leading to excessive production of proinflammatory cytokines and receptor activator of nuclear factor kappa B ligand (RANKL) may cause activation of osteoclasts resulting in bone loss (30). In turn, the interaction between inflamed gingiva, dental plaque and the host response to it may affect fibrotic process in the gingiva.

In conclusion, at present, an effective management of patients with gingival fibromatosis clearly requires the active involvement of both dental and medical professionals to minimize the possibility of complications. Since the pathogenesis of gingival enlargement is not well understood, treatment is still largely limited to maintaining an improved level of oral hygiene, professional scaling, root planning and the surgical excision of overgrown tissue (24, 49). Non-surgical treatment is a far less invasive technique than surgical approaches and it is considered to be the first treatment option for GF. However, such a mode is
insufficient in a relatively prevalent group of patients with diffuse and/or recurrent gingival overgrowth. The long-term benefits of surgery, particularly in the case of these patients, cannot be predicted and they are exposed to the risk of consecutive surgical procedures within relatively short time intervals, which may affect the quality of a patient's life. It has been shown that conventional gingivectomy with laser excision resulted in a better treatment outcome with a reduced rate of recurrence when compared to conventional gingivectomy with flap surgery (50). Although this mode of treatment seems to be more effective than routine surgery, the method still remains highly invasive for the patient. Therefore, since the sole mode of treatment of GF consists of invasive, surgical methods and the long-term benefit of surgery in a number of patients cannot be predicted, discerning studies testing novel tools that might be of value in the future (alternative or supporting) treatment of gingival overgrowth are of great importance. Finally, we report in the present study that the chimeric IgG variant appears to be an effective tool in counteracting collagen fibril accumulation in a fibroblast model derived from diffuse, hereditary gingival fibromatosis. Notably, a novel anti-collagen IgG was effective in culture of cells that derive from gingival lesion with a high propensity to recurrence (high proliferation index). Employing cell cultures from standardized group of patients with recurrent HGF as well as standardising relevant 3D (tissue-like) models will be crucial for further tests of the antibody.

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