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Verapamil inhibits interleukin-6 and vascular endothelial growth factor production in primary cultures of keloid fibroblasts

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Summary An increased secretion of cytokines and growth factors has been hypothesised to play a role in the abnormal growth of keloid fibroblasts. The aim of this study was to evaluate the effect of the calcium antagonist verapamil on the interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) secretion, as well as on cellular growth, in primary cultures of fibroblasts derived from the central part of keloid lesions. These cells grew faster than peripheral keloid and nonkeloid fibroblasts, and, in long-term cultures, became stratified assuming a three-dimensional structure. Compared with peripheral and nonkeloid fibroblasts, central keloid fibroblasts presented an increased production of both IL-6 and VEGF ($P < 0.03$ and $P < 0.005$, respectively). Verapamil (100 μM) decreased IL-6 and VEGF production ($P < 0.03$ and $P < 0.005$, respectively) in central keloid fibroblasts cultures at 72 h. Moreover, verapamil decreased cellular proliferation by 29% and increased apoptosis to an absolute value of 8%. The results of this study demonstrate that in primary cultures of central keloid fibroblasts verapamil reduces the sustained basal IL-6 and VEGF production and inhibits cell growth; these data may offer the link with the beneficial effect of calcium antagonists on keloid scars in vivo.

Keloids are the result of a dysregulated process of wound healing, with proliferation of scar tissue beyond the boundaries of the inciting wound.[1,2] The basic mechanism involved in keloid development is still unknown. Some evidence indicates that excessive matrix accumulation and cell proliferation are distinctive features of keloid lesions.[2–4] Increased proliferation and lower rate of apoptosis have been observed in keloid fibroblasts, suggesting that mechanisms controlling cell proliferation or intercellular matrix production may be altered in keloids.[2–5] Aberration in signaling between stroma and epidermis may underlie abnormalities that contribute to the excessive matrix deposition and fibrosis characteristic of keloid lesions.[6] An important role may be played by cytokines released during normal or pathological wound healing.[7] For

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example, in cultured fibroblasts from keloids interleukin 6 (IL-6) release has been shown to be increased,[8] and vascular endothelial growth factor (VEGF) production to be stimulated by hypoxia,[9] suggesting a possible role for these cytokines in the development of abnormal matrix accumulation or cell proliferation of keloid lesions.

Medical therapy of keloids is still disappointing.[10] The calcium-antagonist verapamil (VP) has been shown to have some beneficial effect in the control of cell growth and matrix accumulation of keloids,[11–13] but the precise mechanism of action is still unknown. The aim of this study was to evaluate the effect of VP on cell growth and cytokines production in fibroblast primary cultures obtained from human keloid tissues.

Material and methods

Origin of cell cultures

The protocol of the study was approved by the ethical committee of our institution and informed consent was obtained from all subjects. Peripheral and central fractions were isolated from tissue explants obtained from patients who underwent plastic surgery for keloids and were used for establishing distinct primary cultures, according to Luo et al.[5] Skin specimens were also obtained during surgery from age-matched nonkeloid-forming subjects and used to set normal fibroblast primary cultures. Samples were minced and placed directly in different plastic culture dishes for incubation with the appropriate medium in a humidified tissue incubator (37 °C, 5% CO_2). Peripheral and central fractions were isolated from keloid tissues, and used for establishing distinct primary cultures, according to Luo et al.[5] Fibroblasts were allowed to proliferate in Dulbecco modified minimal Eagle medium (DMEM) supplemented with L-glutamine, 10% foetal bovine serum (FBS) and antibiotics (Gibco-BRL, Life Technology, Milan, Italy). For hypoxia, cultures were incubated in a hypoxia incubator with a gas mixture containing 5% O_2 and 5% CO_2 balanced with nitrogen. Four cell strains from primary tissues at the second passage were used in the experimental protocol.

Cellular growth curve

Cell replication was investigated in 4×10^4 cell placed directly in plastic culture dishes of 60 mm in DMEM, supplemented with 10% FBS. At fixed intervals (24–48–96–120 h, and 6, 7, 8, 9 and 12 days), the plastic culture dishes were trypsinised in triplicate for each experimental point and the number of cell were counted in a Neubauer Chamber.

Cell proliferation assay

Cell proliferation was evaluated with the tetrazolium salt (MTT) method. Proliferation kit (Roche Diagnostics GmBh, Mannheim, Germany) is a colorimetric assay for the nonradioactive quantification of viable cells, because only metabolically active cells cleave it to form a formazan dye (UV absorbance spectrum is between 550 and 600 nm). Cells were seeded in microtitre plates in final volume of 100 μl complete culture medium at a concentration of 2×10^3 cell/well and grown for 24 h at 37 °C in 5% CO_2. Cells, starved for 24 h in MEM without FBS, were incubated in 1% FBS-supplemented MEM with verapamil (VP, 100 μM) or solvent (control cells) for 96 h. Then 10 μl MTT solution were added to each well and plates were incubated for 4 h. Ten microliters of solubilisation solution were added to each well and plates were kept overnight in the incubator. Absorbance was read at 550 nm using a microtiter plate reader.

Apoptosis detection

The in situ cell death detection kit (TUNEL, Roche Diagnostics GmbH, Mannheim, Germany) was used to detect apoptosis and to quantify DNA strand breaks in individual cells. The cell monolayers were grown directly on sterilised slides (Superfrost, Carlo Erba, Milan, Italy), starved for 24 h in MEM without FCS and then incubated in 1% FCS-supplemented with VP (100 μM) or solvent (control cells) for 96 h. The slides were then fixed in buffered paraformaldehyde, permeabilised with Triton-x, and labelled with TUNEL reaction mixture according to the manufacture's instruction. Samples were analysed using a Leitz Diaplan microscope (Leica, Milan, Italy) equipped with epifluorescence. A negative control (obtained by incubating a slide with labelled solution without terminal transferase) and a positive control (obtained by treating a slide with Dnase I solution) were included in each assay run.

Production of IL-6 and VEGF

Fibroblasts in monolayers were trypsinised and placed in multiwells (6 wells) at a concentration of 2×10^4 with DMEM-supplemented with 10% FBS. For VEGF evaluation experiments were performed in hypoxic conditions, placing cell cultures in a hypoxia incubator with a gas mixture containing 5%

O_2 and 5% CO_2 balanced with nitrogen. After 24 h the medium was changed with MEM-supplemented with 1% FBS. Cytokine production was measured with ELISA using commercially available kits (VEGF, R&D Systems, Milan, Italy; IL-6, Bender MedSystems Diagnostics GmbH, Vien, Austria).

Statistical analysis

All experiments were performed in triplicate on four cell strains from primary tissues at the second passage. Results are presented as mean ± SE. Statistical analysis of results was performed by ANOVA for repeated measures with Bonferroni correction. A value of $P < 0.05$ was considered significant.

Results

The fibroblasts derived from the central part of keloids grew faster than control fibroblasts obtained from patients without evidence of keloid formation (Fig. 1). Keloid fibroblasts showed a significant increase of growth velocity from 96 h onwards ($P < 0.02$ - $P < 0.005$). Kept in a long term cultures (3 weeks), they continued to grow after the confluence; moreover, they looked more tapered and showed extroversions (Fig. 1, inset A), and became stratified showing a three-dimensional structure due to the aggregation of the extracellular matrix (Fig. 1, inset B).

Compared with peripheral and nonkeloid fibroblasts, central keloid fibroblasts presented an increased production of both IL-6 (Fig. 2, upper panel) and VEGF (Fig. 2, lower panel) at 72 hours ($P < 0.03$ and < 0.005, respectively). Central keloid fibroblasts treated with VP exhibited a significant decrease of IL-6 (291 ± 37 pg/ml/well, $P < 0.03$) and VEGF (681 ± 45 pg/ml/well, $P < 0.005$) release at 72 h (Fig. 2). By contrast, verapamil (100 μM) did not cause any significant change in the basal release of both IL-6 and VEGF at 72 h when added to cultures of peripheral (201 ± 73 and 323 ± 80 pg/ml/well, respectively) or nonkeloid fibroblasts (212 ± 65 and 317 ± 78 pg/ml/well, respectively).

Treatment with VP of fibroblasts derived from the central part of keloids caused a significant slowing of cellular growth at 96 h ($P < 0.04$), with complete cessation at 120 h (Fig. 3). At 96 h VP reduced cellular proliferation by 29%, while apoptosis increased to the absolute value of 8% (Fig. 3, insets). There was no significant effect of verapamil on both cellular proliferation and apoptosis in cultures of peripheral fibroblasts or nonkeloid fibroblasts (data not shown).

Discussion

Different types of fibroblasts are present in keloids: one population (peripheral fibroblasts) presents proliferation rate similar to cells derived from normal subjects, while another population (central fibroblasts) shows altered growth and survival, characterised by an abnormal balance between

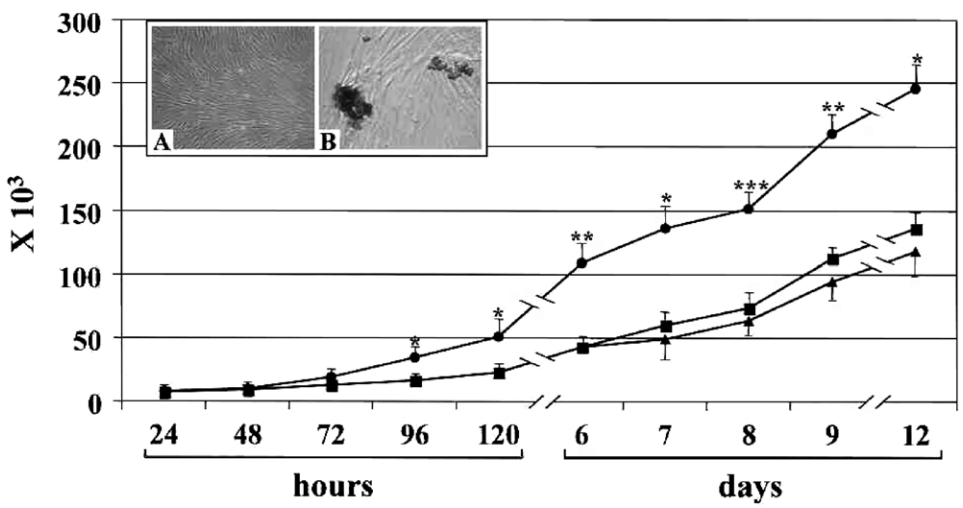

Fig. 1 Growth curve (4×10^4) of fibroblasts from central (●) and peripheral keloid tissues (■), and control fibroblasts from patients without evidence of keloid formation (▲). Values represent the mean ± SE of four independent experiments performed in triplicate. Central keloid fibroblasts showed a significant increase of growth velocity starting from 96 h and afterwards (*$P < 0.02$, **$P < 0.01$, ***$P < 0.005$). They looked more tapered and showed extroversions (inset, A); kept in a long term cultures (3 weeks), they became stratified showing a three-dimensional and united structure due to the confluence of the extracellular matrix (inset, B).

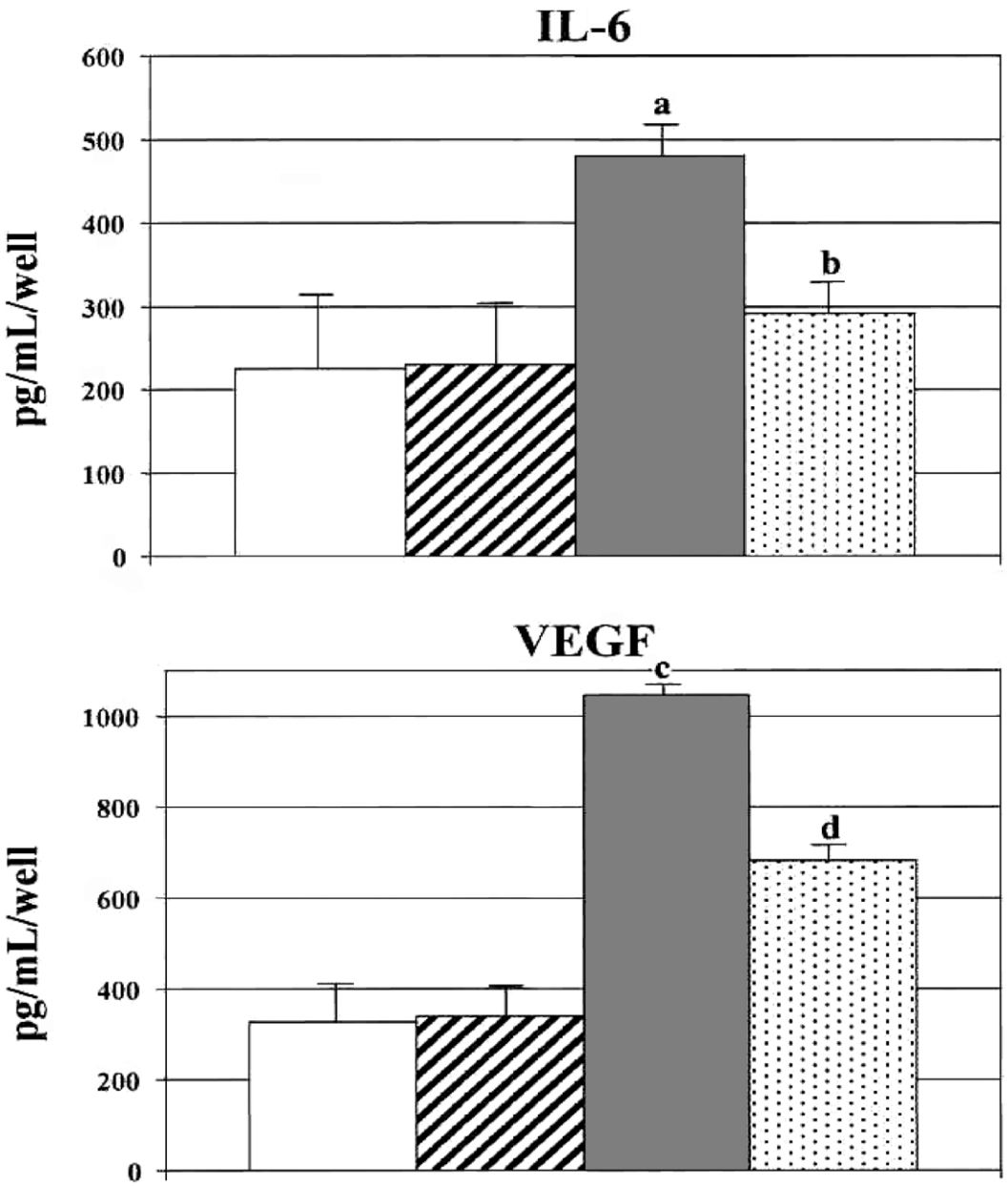

Fig. 2 IL-6 and hypoxia-induced VEGF production in fibroblasts from keloid specimens and control tissues. Each column represents the mean ± SE levels at 72 h of four separate experiments in triplicate. Compared with peripheral (open bars) and nonkeloid (stripped bars) fibroblasts, central keloid fibroblasts (solid bars) presented an increased production of both IL-6 (a, $P < 0.03$) and VEGF (c, $P < 0.005$); central keloid fibroblasts treated with 100 μM VP (dotted bars) showed a decrease of IL-6 (b, $P < 0.03$) and VEGF (d, $P < 0.005$) release.

proliferation and apoptotic cell death. The latter population of fibroblasts is thought to be responsible for the unrestrained growth of the keloid lesion.[5] We have shown that central fibroblasts from keloids are characterised by accelerated growth and are able to secrete increased amounts of IL-6 and VEGF in vitro confirming previous findings.[5,8,9] The novel findings of the present study are that VP treatment of cultured central keloid fibroblasts inhibits growth, induces apoptosis, and decreases cytokine production.

Although calcium-channel blockers are proposed as local therapy in keloids, their mechanism of action is still unknown.[10–13] There is evidence that the calcium channel-blocker VP regulates extra cellular matrix production acting on the synthesis or degradation of proteins.[14–16] VP inhibits proline incorporation[14] and induces pro-collagenase synthesis and collagen degradation[15,16] in keloid fibroblasts. Changes in the cytosolic calcium ion concentration may influence the cellular signalling and fibroblast response to interaction with

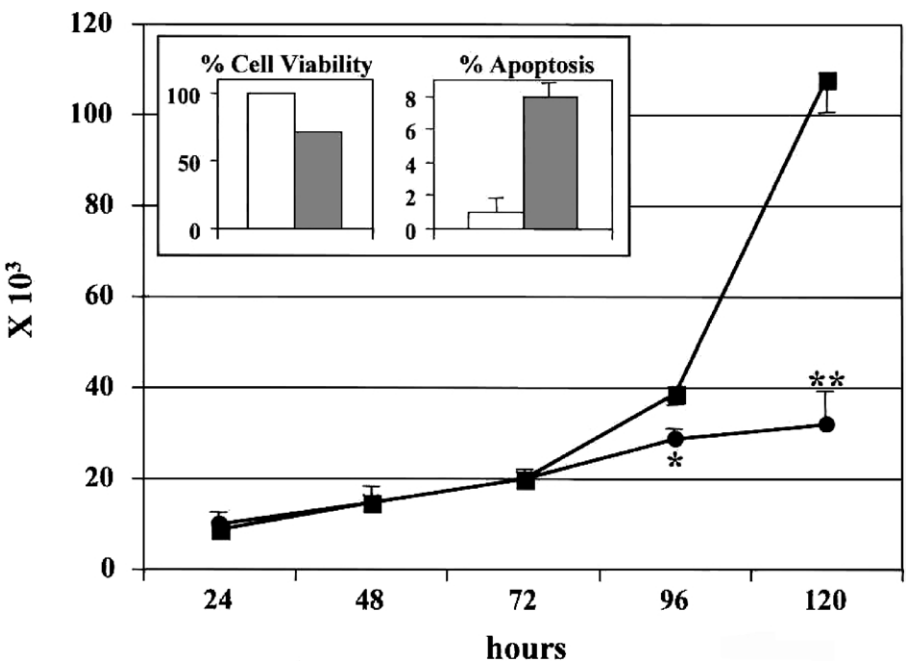

Fig. 3 Effects of VP (●) or solvent (■) on the proliferation of central keloid fibroblasts. Values represent the mean ± SE of four independent experiments performed in triplicate. VP caused a significant slowing of cellular growth at 96 h (*$P < 0.04$), with a stop at 120 h (**$P < 0.001$). Insets show reduced cell viability (evaluated by MTT) and increased apoptosis in VP-treated (solid bars) respect to solvent-treated (open bars) central keloid fibroblasts.

extracellular matrix, and may alter cell shape and proliferation.[16-18] Our findings demonstrate that VP acts on cell growth and apoptosis, suggesting an important role of intracellular calcium in the modulation of these events. Moreover, we also showed that VP may also influence the production of cytokines, such as VEGF and IL-6. A toxic effect of verapamil in our primary cultures of fibroblasts can be excluded by the demonstration that the calcium-antagonist had no effect on cytokine production and cellular proliferation in cultures of both peripheral and nonkeloid fibroblasts.

Several cytokines and growth factors have been identified and are known to be essential for normal wound healing.[7] Altered levels of some cytokines have been associated with abnormal wound healing, both impaired or excessive.[7-9] IL-6 is detected in cutaneous wounds, and increased expression of the IL-6 gene and IL-6 protein have been demonstrated in fibroblasts derived from patients with keloids.[7,8] IL-6 is involved in the growth and differentiation of several cell types, but its role in wound repair is unclear.[19] IL-6 deficient transgenic mice (IL-6 KO) displayed significantly delayed cutaneous wound healing compared with wild-type control animals.[20,21] IL-6 administration seems to be mitogenic for keratinocytes from IL-6 deficient mice.[22] Therefore, IL-6 may act as a differentiation and proliferation signal on dermal fibroblasts and other cell components (keratinocytes, macrophages and so on) during keloid development.

Present and previous findings[9] show that keloid fibroblasts are able to produce sustained levels of VEGF in vitro under hypoxia. VEGF is known to be a potent endothelial cell-specific cytokine, being mitogenic and chemotactic for endothelial cells in vitro, and inducing angiogenesis in vivo.[23,24] VEGF improves granulation-tissue formation in both normal and hypoxic tissues during experimental wounding.[7,23-26] It is released primarily by keratinocytes but also by macrophages and fibroblasts, and is influenced by local tissue conditions and also by nitric oxide production.[25,26] Increased levels of this growth factor may be responsible of abnormal proliferation of central keloid fibroblasts.

In conclusion, central fibroblasts from keloids produce increased amounts of both IL-6 and VEGF, and VP reduces the production of these cytokines in vitro. The results of the present study suggest that VP acts not only on matrix deposition, but also on cell growth and survival, modulating growth factors and cytokines involved in the mechanisms responsible for abnormal growth phenotype typical of keloid scars. Moreover, our findings seem to give a mechanistic explanation for the clinical effect of VP in the treatment of keloid lesions,[11-13] and open the way to controlled clinical trial for evaluating its efficacy and safety in the management of keloids.

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