ORIGINAL ARTICLE

Effects of Copper Tripeptide on the Growth and Expression of Growth Factors by Normal and Irradiated Fibroblasts

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Objective: To evaluate the effects of copper tripeptide (GHK-Cu) on the growth and autocrine production of basic fibroblast growth factor, transforming growth factor β1, and vascular endothelial growth factor by normal and irradiated fibroblasts in a serum-free in vitro environment.

Methods: Primary human dermal fibroblast cell lines were established after explantation from intraoperative specimens obtained from patients who had undergone radiation therapy for head and neck cancer. Normal and irradiated fibroblasts were propagated in serum- and growth factor–free media. Treatment groups were exposed to GHK-Cu (1×10^{-9} mol/L). We measured cell counts and production of basic fibroblast growth factor, transforming growth factor β1, and vascular endothelial growth factor.

Results: Irradiated fibroblasts survived and replicated in serum-free media. The population-doubling times of normal and irradiated fibroblasts exposed to GHK-Cu were faster than those of nontreated controls. Irradiated fibroblasts treated with GHK-Cu doubled at a rate that approximated that of untreated controls, and produced significantly more basic fibroblast growth factor and vascular endothelial growth factor than untreated controls early after GHK-Cu exposure.

Conclusions: Irradiated fibroblasts survive and replicate in serum-free media, establishing this model as ideal for evaluating growth factor production in vitro. Copper tripeptide accelerates the growth of normal and irradiated fibroblasts to the point where treated irradiated fibroblasts approximate the population-doubling time of normal controls. An early increase in basic fibroblast growth factor and vascular endothelial growth factor production by GHK-Cu–treated irradiated fibroblasts may improve wound healing.

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A PRECISE KNOWLEDGE OF wound healing is central to the science of surgery. Wound healing is a sequential cascade of overlapping processes, which occur in a careful, regulated, and reproducible fashion. Tissue injury initiates bleeding, coagulation, inflammation, cell replication, angiogenesis, epithelialization, and matrix synthesis.

Growth factors contribute to wound healing by controlling the proliferation and migration of cells that modulate epithelialization, angiogenesis, and collagen metabolism. Growth factors are produced by a variety of cells, including fibroblasts. By manipulating various growth factors, it may be possible to modify the wound healing process in different clinical states. Two of the major growth factors that have a direct effect on wound healing are fibroblast growth factor (FGF) and transforming growth factor β1 (TGF-β1). One of the major growth factor proteins that has an angiogenic effect is vascular endothelial growth factor (VEGF).

The FGF family consists of 2 closely related isoforms, basic (bFGF) and acidic (aFGF). Basic FGF is a single-chain polypeptide (16.5-18.2 kDa) known for its mitogenic, chemoattractant, regulatory, and angiogenic abilities.[1,2] It is found in a wide variety of cell types and, of all known growth factors, has the broadest range of target cells by influencing all of the diverse cells in wound healing, ie, capillary endothelial cells, fibroblasts, glial cells, neuronal cells, osteoblasts, and myoblasts.[2] In general, most cells are much more sensitive to bFGF than aFGF. Basic FGF has been found to be 10- to 30-fold more active than aFGF and can maximally stimulate proliferation of some cells at 1 ng/mL.[2,3] Basic FGF is stored as an inactive peptide in the extracellular matrix, integrated within the basement membrane. It functions locally and is released when injury has occurred.[1,3]

Transforming growth factor β1 is a homodimeric structure with 2 disulfide-linked polypeptide chains of 12.5 kDa. It is a member of a family of pleiotropic growth factors and is produced by platelets, macrophages, fibroblasts, and smooth muscle cells. Transforming growth factor β1 was originally characterized by its abil-

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ity to reversibly induce the transformed phenotype in certain nontumorigenic cells.[4] At present, TGF-β1 is known to elicit a variety of biological activities and has been shown to dramatically increase the expression of several collagen types in cultured fibroblasts.[5] It has several proinflammatory and regulatory influences, including cell migration, cell cycle progression, extracellular matrix production, and granulation tissue formation.[6-9] In vivo models have shown augmentation of wound healing with topical and intravenous administration.[10,11]

Vascular endothelial growth factor is a homodimeric, heparin-binding glycoprotein (45 kDa).[12-14] It is produced by many cells, including fibroblasts. It has a strong mitogenic influence on endothelial cells and can increase vascular permeability. It also causes vasodilation via stimulation of nitric oxide synthase in endothelial cells. Vascular endothelial growth factor can also increase cell migration and inhibit apoptosis. It plays a significant role in the growth and survival of endothelium and is largely responsible for the angiogenesis associated with wound healing.[15-18]

Radiation therapy is an important treatment modality for cancer of the head and neck; however, irradiated tissues undergo permanent and detrimental cellular changes with significant implications for wound healing. Ionizing radiation causes endothelial cell injury, with endarteritis resulting in atrophy, fibrosis, and delayed tissue repair. The prominent histopathologic finding in all irradiated tissues is a progressive obliteration and decrease in the number of capillaries.[19,20] Also seen is a gradual increase in the amount of fibrous tissue. Clinically, wound healing in irradiated tissues can prove to be difficult, leading in many cases to poor outcomes with respect to form and function.

Modulators are external agents that alter the autocrine growth factor milieu and thus have the potential to augment the wound healing mechanism. Certain modulators may offer clinicians a means of optimizing wound healing. Once a modulator's autocrine growth factor stimulatory properties are known, it could be placed into a wound to achieve the desired healing response clinically. By applying various modulators to irradiated dermal fibroblasts grown in serum-free media, we can evaluate the downstream effects on production of these growth factors (bFGF, TGF-β1, and VEGF) in vitro.

The copper tripeptide complex glycyl-L-histidyl-L-lysine-Cu^{2+} (GHK-Cu) is a naturally occurring tripeptide. It was first isolated from human plasma, but can be found in saliva and urine. During wound healing, it may be freed from existing extracellular proteins via proteolysis. In the wound environment, it serves as a chemoattractant for inflammatory and endothelial cells.[21] It naturally occurs at a concentration of 200 ng/mL (10^{-7} mol/L) by 20 years of age. By 60 years of age, the concentration declines to 80 ng/mL. Cell culture studies have shown that GHK-Cu has maximal biological effect at 10^{-9} mol/L.[22,23] At this concentration, GHK-Cu has been shown to increase messenger RNA production for collagen, elastin, proteoglycans, and glycosaminoglycans in fibroblasts.[3,22-24]

Copper tripeptide has been shown to have significant clinical application in the field of wound healing and tissue repair. It has been demonstrated that GHK-Cu stimulates cultured normal fibroblasts to synthesize collagen and induces a dose-dependent increase in the synthesis of glycosaminoglycans.[21,22,25] It has also been shown that extracellular matrix accumulation increases in the rat wound model as a result of GHK-Cu application.[26] Clinical studies[27,28] have demonstrated the efficacy of GHK-Cu preparations in facilitating an increased rate of wound healing in diabetic ulcers and in patients who have undergone Mohs surgery. Copper tripeptide induces angiogenesis in rabbit models at 10^{-12} mol/L by acting as a chemoattractant for capillary cells.[23] A recent study from our laboratory has shown that GHK-Cu may improve the autocrine growth factor milieu in keloid fibroblasts.[29] It is unknown how this compound affects irradiated fibroblasts.

METHODS

Normal and irradiated fibroblasts were obtained directly from operative specimens and established as primary cell line cultures. Use of these specimens was approved by the Human Subjects Committee at Stanford University, Stanford, Calif. Operative specimens contained cervical skin exposed or not exposed to 5000 rad (5000 cGy) of external beam radiation. With the use of sterile technique under a laminar flow hood, the dermis of each gross specimen was isolated and minced into 1- to 2-mm^3 fragments. An antimicrobial wash was performed using a combination of 5% penicillin, streptomycin sulfate, and amphotericin B (GIBCO, Grand Island, NY) in Dulbecco phosphate-buffered saline solution (GIBCO). The specimens were then placed in 10 mL of culture medium (10% fetal calf serum in Dulbecco modified eagle medium with 1% L-glutamine and a combination of 1% penicillin, streptomycin sulfate, and amphotericin B) (GIBCO) in crosshatched 75-cm^2 tissue culture flasks (Falcon; Becton-Dickinson, Franklin Lakes, NJ). Flasks were then incubated at 37°C in a humidified 5% carbon dioxide atmosphere. The medium was changed every few days.

An explant technique was used to establish cell lines. The flasks were monitored for fibroblast cellular outgrowth by means of phase-contrast microscopy. When sufficient outgrowth of fibroblasts occurred, the flasks were washed with phosphate-buffered saline solution to remove nonadherent cells. The remaining adherent fibroblasts were released using 0.05% trypsin (GIBCO) phosphate-buffered saline solution, subcultured, and passed into 75-cm^2 culture flasks. The culture medium was changed every few days, and successive cultures were passed at confluence.

Experiments were performed with cells in their first or second passage. At the time of experimentation, the fibroblasts were washed with phosphate-buffered saline solution, and 0.05% trypsin was used to release the confluent cells from the flask wall. Trypsin soybean inhibitor (GIBCO) in a 1:1 ratio inactivated the trypsin. Cell culture viability was determined by means of trypan-blue dye exclusion, and cells counts were performed in duplicate using a hemocytometer and phase-contrast microscopy. Cells were then seeded at a density of 5×10^3 (normal) and 3×10^3 (irradiated) cells/well in each well of a sterile 96-well plate (Falcon; Becton-Dickinson) using a commercially available serum-free medium (UltraCULTURE; Biowhittaker, Walkersville, Md). This medium has been shown to sustain dermal fibroblast growth to at least 7 days with greater than 90% viability.[4,30-32]

At 0 hours, GHK-Cu solution (1×10^{-9} M) in the serum-free medium was added to the treatment groups, and an equal volume of plain serum-free medium was added to the untreated control groups. Untreated cells from each cell line were used for controls. Cell counts were performed using a cell proliferation assay system with reagent 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) (Boeh-

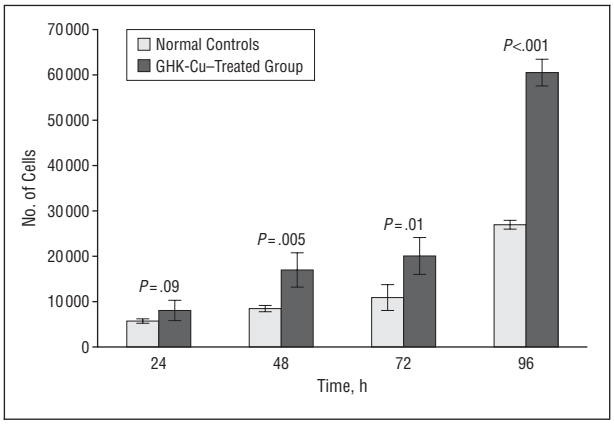

Figure 1. Growth comparison within a cell line (fibroblasts treated with copper tripeptide [GHK-Cu] vs normal controls). Normal fibroblasts showed significantly different growth measurements at 48, 72, and 96 hours ($P=.005$, $P=.01$, and $P<.001$, respectively). Limit lines indicate standard deviation.

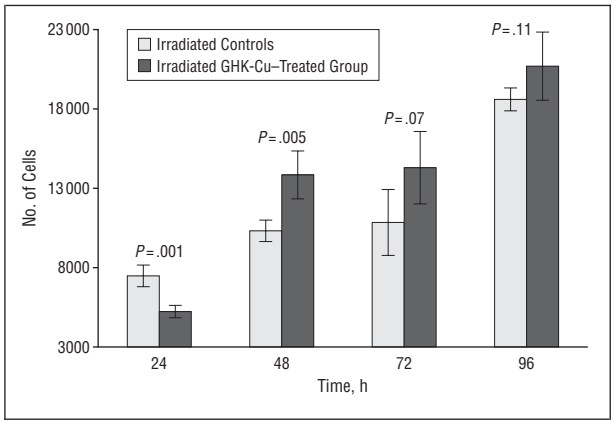

Figure 2. Growth comparison within a cell line (irradiated fibroblasts treated with copper tripeptide [GHK-Cu] vs irradiated controls). Irradiated fibroblasts showed significantly different growth measurements at 24 and 48 hours, with a trend toward more growth in the treated group at 72 and 96 hours ($P=.001$, $P=.005$, $P=.07$, and $P=.11$, respectively). Limit lines indicate standard deviation.

ringer Mannheim, Indianapolis, Ind) 24, 48, 72, and 96 hours after initiation for growth curve generation. The WST-1 assay is a colorimetric assay used in the quantification of cell proliferation and cell viability based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. It is a nonradioactive alternative to the tritium-thymidine incorporation assay. Assays were read using an automated plate reader (ELx800; Bio-Tek Instruments, Inc, Winooski, Vt). Optical densities were analyzed with commercially available software (KC4; Bio-Tek Instruments, Inc). Cell counts were determined by comparison with a standard curve derived from known cell quantities calculated for each cell type and medium.

At each 24-hour interval, cell-free supernatant was collected from the testing wells in triplicate. Samples were stored at –80°C in microcentrifuge tubes for later growth factor assays. Expression of bFGF, TGF-β1, and VEGF was evaluated for each group by means of a solid-phase enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn) at 24-hour intervals. We calculated cell population-doubling times (PDT) from logarithmic best-fit curves.

We performed data analysis using commercially available software (Microsoft Excel for Windows 2002; Microsoft Corporation, Redmond, Wash). For statistical analysis, we used the methods described by Glantz.[33] We assessed statistical differences between groups using the paired *t* test and considered differences significant at $P<.05$.

RESULTS

CELL GROWTH

All groups survived and replicated over time in serum-free media. Comparisons were made within cell lines (GHK-Cu–treated vs controls). Normal fibroblasts showed significantly different growth measurements at 48, 72, and 96 hours ($P=.005$, $P=.01$, and $P<.001$, respectively) (**Figure 1**). Irradiated fibroblasts showed significantly different growth measurements at 24 and 48 hours, with a trend toward more growth in the treated group at 72 and 96 hours ($P=.001$, $P=.005$, $P=.07$, and $P=.11$, respectively) (**Figure 2**). The PDTs were faster in the GHK-Cu–treated groups. Modulation with GHK-Cu affected irradiated fibroblasts such that their PDT approximated that of normal controls (**Figure 3**).

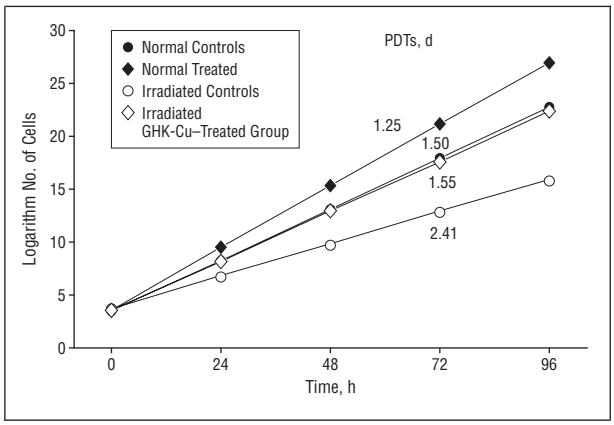

Figure 3. Population-doubling times (PDTs) measured as best-fit linear equation to log growth curves. The PDTs were faster in the copper tripeptide (GHK-Cu)–treated groups. Modulation with GHK-Cu affected irradiated fibroblasts, such that their PDT approximated that of normal controls.

BASIC FGF

We compared overall bFGF production within cell lines (GHK-Cu–treated vs controls). Normal fibroblasts produced the same amount of bFGF, except at 96 hours, when the normal control group produced more ($P=.01$). By 120 hours, the overall production was equivalent ($P>.99$). The GHK-Cu–treated irradiated fibroblasts showed significantly greater production of bFGF than irradiated controls at 24 and 72 hours ($P=.03$ and $P=.03$, respectively). By 120 hours, the overall production was equivalent ($P=.82$).

We compared per-cell bFGF production between cell lines (normal controls vs irradiated controls). Normal controls produced significantly more bFGF at 24, 48, 96, and 120 hours ($P<.001$, $P=.01$, $P=.04$, and $P=.03$, respectively) (**Figure 4**). The GHK-Cu–treated irradiated fibroblasts produced significantly more bFGF than normal controls at the 24-hour interval ($P=.03$) (**Figure 5**).

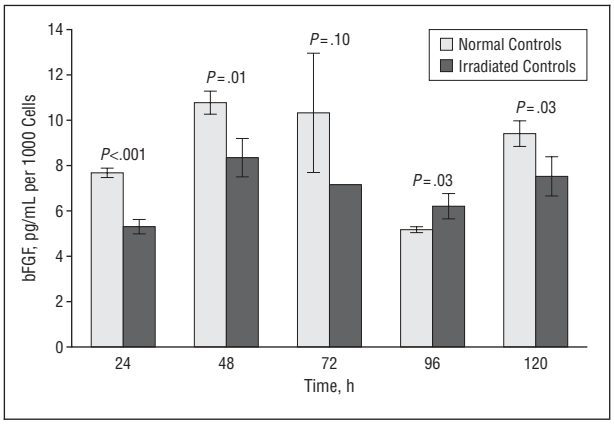

Figure 4. Basic fibroblast growth factor (bFGF) production per cell between cell lines (normal vs irradiated controls). Normal controls produced significantly more bFGF at 24, 48, 96, and 120 hours ($P<.001$, $P=.01$, $P=.04$, and $P=.03$, respectively). Limit lines indicate standard deviation.

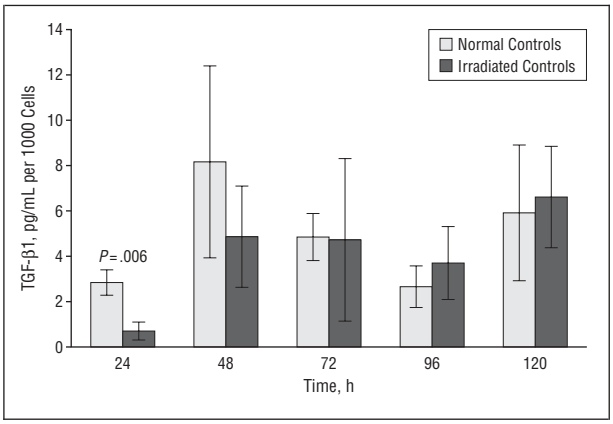

Figure 6. Transforming growth factor β1 (TGF-β1) production per cell between cell lines (normal vs irradiated controls). Normal controls produced significantly more TGF-β1 at 24 hours ($P=.006$). Limit lines indicate standard deviation.

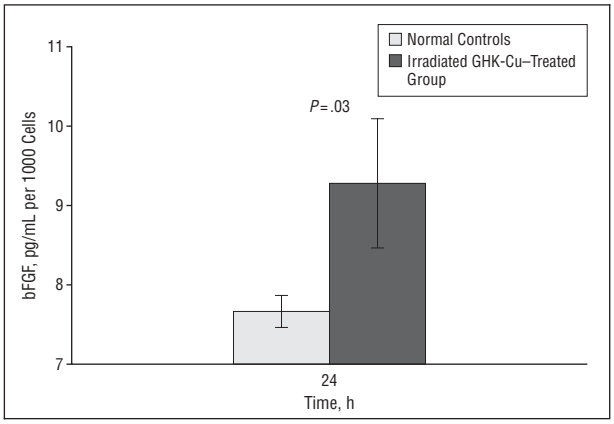

Figure 5. Basic fibroblast growth factor (bFGF) production per cell between copper tripeptide (GHK-Cu)–treated irradiated fibroblasts and normal controls at the 24-hour interval ($P=.03$). Limit lines indicate standard deviation.

TRANSFORMING GROWTH FACTOR β1

We compared overall TGF-β1 production within cell lines (GHK-Cu–treated vs controls). Normal fibroblasts produced equivalent amounts of TGF-β1 at all time intervals. Irradiated fibroblasts also produced equivalent amounts of TGF-β1 at all time intervals.

We compared TGF-β1 production per cell between cell lines (normal controls vs irradiated controls). Normal controls produced significantly more TGF-β1 at 24 hours ($P=.006$) (**Figure 6**).

VASCULAR ENDOTHELIAL GROWTH FACTOR

We compared VEGF production within cell lines (GHK-Cu–treated vs controls). Normal controls produced more VEGF than the treatment group at 24, 48, and 96 hours ($P<.001$, $P=.03$, and $P=.01$, respectively). By 120 hours, the overall production was equivalent ($P=.93$). Irradiated fibroblasts produced equivalent amounts of VEGF at all time intervals.

We compared VEGF production per cell between cell lines (normal controls vs irradiated controls). Normal controls produced significantly more VEGF at 24 and 48 hours ($P=.01$ and $P=.055$, respectively) (**Figure 7**). Treated irradiated fibroblasts produced significantly more VEGF than normal controls at the 24-hour interval ($P=.047$) (**Figure 8**).

COMMENT

Several interesting findings are demonstrated. First, survival and growth of irradiated fibroblasts was demonstrated within the serum-free media. To our knowledge, our laboratory is the first to document this phenomenon using irradiated human fibroblasts. Our laboratory has already demonstrated survival and growth of normal, fetal, and keloid fibroblasts in this serum-free environment. Serum-free cell culture is essential when measuring changes in the growth factor milieu and is now a viable model for future studies involving irradiated fibroblasts.

Second, the data established differences in the baseline production of growth factors between normal and irradiated fibroblasts in a head-to-head model. Production of bFGF by normal fibroblasts was significantly increased when compared with that of irradiated fibroblasts at all but 1 time point (72 hours). Production of TGF-β1 by normal fibroblasts was significantly increased when compared with that of irradiated fibroblasts at the 24-hour mark. Finally, production of VEGF by normal fibroblasts was significantly increased when compared with that of irradiated fibroblasts at 24 and 48 hours. It is reasonable to suppose that these differences play an influential role in the differing wound-healing properties of these wounds clinically.

Third, the data show that modulation of the environment with GHK-Cu is associated with changes in the growth factor milieu. The GHK-Cu–treated irradiated fibroblasts showed significantly greater production of bFGF than controls at 24 and 72 hours. In fact, GHK-Cu–treated irradiated fibroblasts produced significantly more bFGF than normal controls at the 24-hour interval. Furthermore, GHK-Cu–treated irradiated fibroblasts produced significantly more VEGF than normal controls at the 24-hour interval. This finding is of importance given the known benefit of an early presence of these growth factors in the healing wound.

Finally, the data show that modulation of the environment with GHK-Cu is associated with a dramatic increase in fibroblast PDT. This was demonstrated in the normal

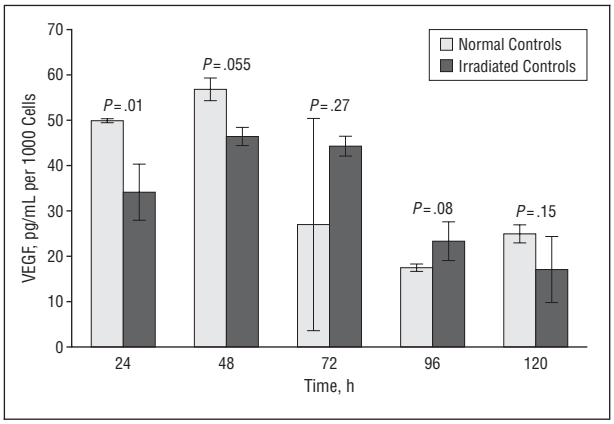

Figure 7. Vascular endothelial growth factor (VEGF) production per cell between cell lines (normal vs irradiated controls). Normal controls produced significantly more VEGF at 24 and 48 hours (P=.01 and P=.055, respectively). Limit lines indicate standard deviation.

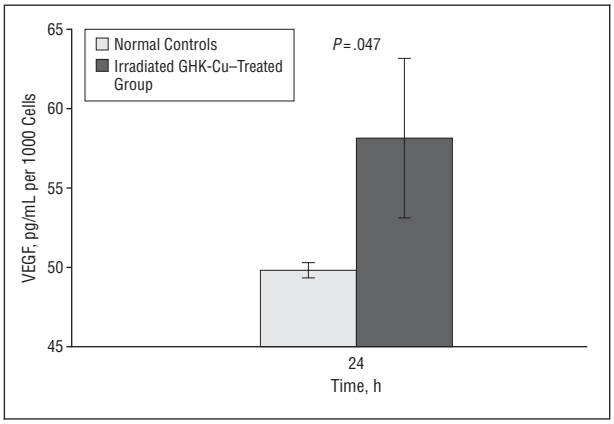

Figure 8. Vascular endothelial growth factor (VEGF) production per cell between copper tripeptide (GHK-Cu)–treated irradiated fibroblasts and normal controls at the 24-hour interval (P=.047). Limit lines indicate standard deviation.

and irradiated cell lines. One striking finding is that population growth in GHK-Cu–treated irradiated fibroblasts assumed that of normal controls. The clinical implications of this are not yet known. However, given the important role of fibroblasts in wound healing, one might hypothesize that more fibroblasts in an irradiated wound bed would lead to a generalized improvement in wound healing.

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