Arecoline increases basic fibroblast growth factor but reduces expression of IL-1, IL-6, G-CSF and GM-CSF in human umbilical vein endothelium

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BACKGROUND: Areca nut chewing is associated with oral submucous fibrosis (OSF). Raised vascular basic fibroblast growth factor may induce fibrosis. Arecoline is a muscarinic alkaloid in areca nut, which we earlier reported causes injury and necrosis of human endothelium.

MATERIALS AND METHODS: Human umbilical vein endothelial cells were exposed to arecoline with or without tumor necrosis factor-α, and separately to acetylcholine, muscarine, or nicotine. Protein levels of basic fibroblast growth factor, as well as the inflammatory cytokines: granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor, and Interleukins-6, 1α and 1β, were determined by enzyme-linked immunosorbent assay. mRNA levels were established by real-time reverse transcription polymerase chain reaction.

RESULTS: Basic fibroblast growth factor was released into the culture medium at arecoline levels causing necrosis (P < 0.05). This contrasted with an opposite effect of arecoline on levels of the inflammatory cytokines (P < 0.05). Tumor necrosis factor-α increased IL-6 and granulocyte-macrophage colony stimulated factor, but arecoline reduced this stimulated expression (P < 0.05). Arecoline had no effect on mRNA for basic fibroblast growth factor, although there was reduced mRNA for the separate inflammatory cytokines studied. The effect of acetylcholine, muscarine, and nicotine was minimal and dissimilar to that of arecoline.

CONCLUSIONS: Data raise the possibility that arecoline-induced, vascular basic fibroblast growth factor contributes to OSF, by combining increased growth factor expression with endothelial necrosis, and thus driving fibroblast proliferation.

Keywords: arecoline; cytokine; endothelium; fibroblast growth factor

Introduction

Areca nut is widely chewed in Asia and in migrant communities throughout the Western world and is often wrapped in betel piper vine to form a quid with or without spices, tobacco, and slaked lime. Areca nut has psychoactive addictive activity and is a group 1 carcinogen (1–3). In addition, areca nut appears responsible for oral submucous fibrosis (OSF), a painful pre-malignant fibrotic condition severely limiting oral opening (4–8).

Arecoline is the major alkaloid in areca nut and has cytotoxic, apoptotic, and proliferative effects (9–13). Altered cultured fibroblast and keratinocyte behavior is reported above 0.1 µg/ml (0.64 µM), and cell death commences at doses of 10–30 µg/ml (64–193 µM) (12–15). We have demonstrated background salivary arecoline levels over the 0.1 µg/ml (0.64 µM) threshold in two-third of habitual areca nut chewers, while levels over 10 µg/ml (64 µM) were seen during chewing in approximately one-third of subjects (16). Maximum salivary arecoline levels during areca nut chewing are above the cytotoxic level and are reported to range from 97 µg/ml (625 µM) (16) to 142 µg/ml (914 µM) (17).

There is very greatly reduced vascularity in OSF relative to normal mucosa (18, 19), and this may reflect at least in part, toxic effects of areca nut components. Separately, we have a long established interest in endothelium in inflammation and wound healing (20–23), and this together with reduced vascularity and blood flow in OSF (18, 19) led us to investigate potential toxicity of arecoline in cultured human
umbilical vein endothelial cells (HUVEC). We observed HUVEC necrosis after 24-h exposure to arecoline at or above 333 μg/ml (2150 μM) and ultrastructural signs of cellular stress by 111 μg/ml (716 μM). However, there was no evidence for arecoline-induced endothelial apoptosis (24). Despite the role of arecoline as a stimulatory and toxic agent, little is known of the endothelial response to arecoline with regard to cytokine synthesis, although reduced NO production by endothelium in umbilical arteries and veins and HUVEC cycle arrest are reported (25).

Fibrotic disorders such as scleroderma, keloid formation, and OSF involve overproduction of extracellular matrix by fibroblasts (4–8, 26). Growth factors and cytokines secreted by inflammatory leukocytes, fibroblasts, and endothelial cells have been suggested as contributing to initiation and progression of connective tissue fibrosis (27). Increased expression of fibrogenic and inflammatory cytokines such as Interleukin-1-α (IL-1α), Interleukin-1-β (IL-1β), Interleukin-6 (IL-6), transforming growth factor-β, basic fibroblast growth factor (bFGF), and platelet-derived growth factor is seen by immunohistochemistry in tissues affected with OSF (28, 29).

Basic fibroblast growth factor has potent proliferative activity for fibroblasts and endothelium, lacks a conventional secretory signal sequence, and is considered important in development of a number of fibrotic conditions including OSF (30–40). Further, bFGF acts synergistically with other growth factors stimulating collagen production by skin fibroblasts (41). Of particular relevance to the current study is correlation between bFGF detected by immunohistochemistry and the severity of OSF (29). We have separately reported elevated bFGF in HUVEC during apoptosis, and interpreted this as a potential negative feedback mechanism for vascular rarefaction (42). This together with the apparent role of bFGF in OSF (29) and the toxic activity of arecoline for endothelium (24) suggested to us the possibility that arecoline may similarly increase bFGF in HUVEC, and this study describes work demonstrating this to be the case.

To provide comparison with bFGF, we also examined the effect of arecoline on expression of a number of inflammatory cytokines made by endothelium, including IL-1, IL-6, granulocyte colony stimulating factor (G-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF), all inflammatory cytokines strongly regulated in HUVEC (43).

Materials and methods

Materials

Cell culture Medium 199 (M199), Hanks balanced salt solution (HBSS), polymyxin B solution, hematoxylin, gelatine, bovine serum albumin (BSA) fraction V, endothelial growth supplement (ECGS), dimethyl sulfoxide, arecoline hydrobromide, nicotine, acetylcholine chloride, muscarine, b-mercaptopoethanol (β-ME), and AR ethanol were purchased from Sigma Aldrich (St. Louis, USA). TNF-α was bought from Chemicon (Billerca, MA, USA). The antibiotics such as penicillin, streptomycin, and the antimycotic amphotericin B were obtained from Life Technologies (Carlsbad, CA, USA). Heparin was bought from Pfizer (New York, NY, USA). Phosphate-buffered saline (PBS) tablets were purchased from Oxoid (Hampshire, UK). Iron-fortified bovine calf serum (BCS) was obtained from Bovogen (Keilor East, Vic., Australia). Trypsin (0.25%)/EDTA (1 mM) was from JRH Biosciences (Lenexa, USA). Cell culture plastic-wares including flasks, cell culture plates, and centrifuge tubes were supplied by Costar (Cambridge, MA, USA), while disposable membrane filters were purchased from Sartorius, Ministart (Gottingen, Germany). Cell scrapers were from Iwaki® Scitech Division (Chiba, Japan), and Triton X100 was purchased from Merk (Darmstadt Germany). ELISA kits for bFGF, IL-1α, IL-1β, IL-6, GM-CSF, and G-CSF detection were in the Duoset® ELISA Development System, which included primary antibodies, secondary antibodies, Streptavidin-Horse Radish Peroxidase (HRP), and substrate reagents and were purchased from R & D Systems (Minneapolis, MN, USA). Sulfuric acid (H2SO4) was acquired from APS Chemicals (Auburn, NSW, Australia). ELISA plates were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA). RNAs-pre Mini Kits for extraction of RNA from HUVEC, PCR strip tubes (0.1 ml) and caps, and nuclease-free water were purchased from Qiagen GmbH (Hilden, Germany). Ambion® RNaseZap® and Platinum® SYBR® Green qPCR SeperMix-UDG, Superscript III reverse transcriptase (200 U/μl), Oligo (dT)20, primers, 10 mM dNTP mix, 10× RT buffer, 25 mM MgCl, 0.1 M DTT, RNase-OUT™ (40 U/μl), and RNase H were purchased from Invitrogen (Carlsbad, CA, USA). Gene-specific primers (bFGF, IL-1α, IL-1β, IL-6, GM-CSF, G-CSF) and primers for housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-Actin] were ordered from Gene-Works Pty Ltd (Hindmarch, SA, Australia).

Isolation and culture of human endothelium

Umbilical cords were obtained from the Westmead Hospital Maternity Unit with informed consent as approved by the Sydney West Area Health service Human Research Ethics Committee. HUVEC were isolated by collagenase perfusion as described elsewhere (44–46) and cultured on gelatine-coated culture surfaces (0.1% gelatine, PBS), in complete culture medium (CM) consisting of medium 199 (80%), BCS (20%), heparin (30 U/ml), and ECGS (40 μ/ml) with the antibiotics such as penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml). Cells cultured by this procedure had the characteristic cobble stone morphology and positive labeling for Factor VIII-associated antigen as well as binding of Ulex europaeus lectin characteristic of HUVEC (44, 47, 48).

Culture conditions for evaluation of endothelial cytokine production

Confluent cultures of HUVEC in passages 4 or 5 were collected from 75 cm² tissue culture flasks using Trypsin/EDTA and seeded at confluence into either 6, 12, or 24 well gelatine-treated tissue culture plates. HUVEC were allowed to attach and incubated for 2–3 days at 37°C, under 5% CO₂ and 95% relative humidity before washing twice with M199 and stimulation for up to 24 h.

Where the effect of arecoline on cytokine antigen levels was studied, HUVEC in 24-well tissue culture plates were stimulated with 500 μl volumes of arecoline at concentra-
tions of: 6440 μM; 2150 μM; 716 μM; 239 μM; 79.6 μM; 26.5 μM; 8.84 μM; 2.95 μM; 0.98 μM; 0.33 μM; and 0 μM in M199 supplemented with 4% BSA. To evaluate the time course of the HUVEC responses to arecoline, cells were stimulated with: 0 μM; 239 μM; or 6444 μM arecoline for increasing times of: 0, 4, 8, 12, 16, and 24 h. To increase relative antigen levels for improved detection in some experiments, 12-well tissue culture plates were used applying 500 μl volumes of CM, and this was performed where the response to arecoline was compared with that to acetylcholine, muscarine, and nicotine applied at 0 M, 10^{-3} M, 10^{-4} M, 10^{-3} M, and 10^{-2} M. Twelve-well tissue culture plates were also used in further experiments where the effect of arecoline on TNF-α stimulated cytokine synthesis was evaluated by inclusion or otherwise of TNF-α at 3 nM, shown in separate preliminary experiments to be maximally stimulatory for HUVEC cytokine production. Cell culture supernatants were collected from HUVEC into 2-ml Eppendorf tubes and centrifuged at 300 g for 2 min to pellet cellular debris, before transfer of the remaining supernatant to fresh Eppendorf tubes. Cell lysates were obtained from the remaining monolayers, which were washed twice with M199 prior to lysis and scraping into 500 μl volumes of Triton X 100 (0.5% in PBS) and collection into separate fresh Eppendorf tubes. Samples were stored at –20°C for later ELISA assay.

Where the effect of arecoline on mRNA expression was studied, HUVEC in fifth passage were seeded at confluence in 2 ml volumes of CM into gelatin-treated six-well tissue culture plates. Following 2–3 days of culture, cells were washed and treated for 24 h with or without arecoline at 716 μM in M199 with BSA (4%), before isolation of mRNA.

**Enzyme-linked immunosorbent assays for IL-1, IL-6, G-CSF, and GM-CSF**

Assays were performed following the manufacturer’s instructions, quantifying cytokines with standard curves established by material provided by the manufacturer. In brief, 96-well ELISA plates were coated with cytokine-specific capture antibodies and allowed to incubate overnight at room temperature. Wells were then washed three times with wash buffer consisting of Tween 20 (0.05% in PBS), before 1 h blocking at room temperature for non-specific binding with 300 μl per well of reagent diluent (1% BSA in PBS). Wells were then washed with wash buffer before application of 100 μl per well of cell supernatants or lysates, while a series of seven separate one in two serial dilutions of standards was also applied in reagent diluent to establish standard curves relative to blank controls. Plates were then sealed and allowed to incubate for 2 h at room temperature. ELISA plates were subsequently washed three times in wash buffer prior to addition of 100 μl/well volumes of biotinylated detection antibodies in reagent diluent at the working dilution and incubation for 2 h at room temperature. Plates were then washed three times with wash buffer, and 100 μl/well Streptavidin-HRP conjugate added and diluted 1:200 in reagent diluent. Plates were then allowed to further incubate for 20 min at room temperature, sealing and covering the plates to avoid direct light. The plates were then washed three times with wash buffer before incubation with substrate solution (100 μl/well) comprising a 1:1 mixture of H2O2 and tetramethylbenzidine for 20 min at room temperature, after first sealing and covering to protect from direct light. Color development was stopped by addition of H2SO4 (1 M) and optical density (OD) determined using a microplate reader (Bio-Rad Model 3550 plate reader, Hercules, CA, USA) at 450 nm and a corrective measurement at 570 nm. Standard curves were constructed, and antigen levels defined as undetectable when OD levels reached levels equivalent to blank controls.

**Isolation of mRNA from HUVEC**

mRNA expression was studied in three separate experiments with HUVEC from three separate donors and isolated according to the manufacturer’s instructions. In brief, each well with HUVEC was washed twice with PBS after collection of supernatants for verification of cytokine production by ELISA, followed by addition of 350 μl Buffer RLT (Qiagen, Hilden, Germany) with β-ME at a ratio of 1:100. Cell lysates were harvested and vortexed prior to mixing with 350 μl of 70% AR ethanol. Samples were then transferred to RNase spin columns for elution of RNA as per the manufacturer’s instructions. The mRNA thus obtained was stored at –20°C and used for Real-time reverse transcription polymerase chain reaction (RT-PCR) before measurement by spectrophotometer. RNA integrity was confirmed using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) prior to quantitation of specific mRNA expression.

**Quantitation of mRNA expression**

Real-time reverse transcription polymerase chain reaction was performed using double-stranded DNA produced by reverse transcription of isolated mRNA. Reverse transcription was performed using the SuperScript™ III First-Strand Synthesis System for RT-PCR according to the manufacturer’s instructions. A minimum of 32 ng mRNA containing 8 μl of RNase-free water was combined with 1 μl 50 μM oligo (dT)20 primer and 1 μl 10 mM dNTP mix in 0.2 ml RNase-free tube before incubation at 65°C for 5 min followed by immediate transfer on ice for at least 1 min. The cDNA synthesis mix was prepared by mixing 2 μl 10× RT buffer, 4 μl 25 mM MgCl2, 2 μl 0.1 M DTT, 1 μl RNSaeOUT™ (40 U/μl), and 1 μl SuperScript™ III RT (200 U/μl) and added to each RNA/primer mixture followed by gentle mixing and collected by brief centrifugation prior to incubation at 50°C for 50 min. Reactions were terminated by incubation at 85°C for 5 min and chilled on ice and collected by brief centrifugation before the addition of 1 μl of RNase H to each tube to degrade mRNA templates from the cDNA:RNA hybrid molecule, and then incubated for 20 min at 37°C. cDNA synthesis reaction products were stored at –20°C to be used for RT-PCR.

Real-time reverse transcription polymerase chain reaction was performed for bFGF, IL-1α, IL-1β, IL-6, GM-CSF, and G-CSF and for the widely accepted housekeeping genes of GAPDH and β-actin. Gene-specific primers were designed using the Universal Probe Library (Roche Applied Science, Indianapolis, IN, USA) Web site and primer 3 Software (SimGene.com, at: http://simgene.com/hosted_tools#PLD) (Table 1). Standard curves were generated for each RT-PCR by mixing the original cDNA from all the samples and 6 further 1:5 dilutions of the pool, while cDNA template of all
samples was diluted 1:50 to be subjected to RT-PCR for differential expression of genes under investigation. RT-PCRs were prepared by mixing Platinum® SYBR Green (5 μl), forward primer (0.3 μl), reverse primer (0.3 μl), and RNase-free water (1.4 μl) and transferring to RT-PCR strip tubes prior to addition of diluted cDNA templates (5 μl) for a total final volume of 12 μl. All the reaction tubes were transferred to a Rotor Gene 6000 PCR machine and run with RT-PCR temperature conditions set as follows: first hold: 50°C for 2 min; second hold: 95°C for 2 min; Cycling: 95°C for 15 s; 60°C for 45 s. Identity of PCR products was confirmed by sequencing by the DNA Sequencing Facility, Westmead Millennium Institute, Westmead, Australia.

**Analysis of cytokine antigen data**

Optical densities of duplicate standards with known concentrations of cytokine were used to produce standard curves for each set of samples assayed, and these data used to generate a formula for each standard curve describing a straight line in the Cartesian plane. Relevant formulae were then used to calculate the concentration of cytokine in individual samples. Most experiments were performed at least six times, with cells from six separate biological donors. The Wilcoxon signed-rank test was used to evaluate statistical significance between different treatment groups across multiple experiments, while the Mann–Whitney U-test was used in considering results within groups across experiments, and Student’s t-test was applied within individual experiments or where data across separate experiments were clearly normally distributed. A P value < 0.05 was considered statistically significant, where appropriate data were expressed as Mean ± SEM.

**Analysis of RT-PCR data**

Real-time reverse transcription polymerase chain reaction data was analyzed using Corbett Rotor-6000 Application Software, version 1.7 (Qiagen GmbH, Hilden, Germany) to obtain the Ct values for each sample. Duplicate values were obtained for each sample for three separate biological donors. Each experiment was repeated multiple times for optimization until reproducible results were obtained. Further analysis for differential gene expression was performed following the ΔΔCT method (49). Due to the limited sample size, and nonparametric distribution of RT-PCR data across different donors, statistical analysis across donors could not be reliably performed and results are shown as average values of expression for each donor.

### Results

**Human umbilical vein endothelial cells-produced bFGF in response to arecoline**

Consistent with the absence of a secretory signal sequence, bFGF antigen was mostly in cell lysates, with only low levels found in culture supernatants (Fig. 1). Levels of bFGF increased in a dose-dependent manner upon HUVEC stimulation with arecoline at concentrations from 26.5 to 716 μM (P < 0.01) (Fig. 1A,B). Arecoline at 2150 μM or above resulted in HUVEC death and this was associated with release of bFGF from cells into supernatants (Fig. 1A, B). Similar results were found in seven experiments with cells obtained from six separate donors.

Considering the time course of bFGF synthesis, when cells were treated with arecoline at 239 μM, there was a significant increase in bFGF levels in lysates by 4–8 h of stimulation (P < 0.05), with little change in antigen level over the remaining 24-h incubation. Stimulation with 6440 μM, however, did not increase total bFGF levels, but instead resulted in release of bFGF from cells into supernatants starting at 8 h and increasing till 24 h (P < 0.05) (Fig. 1C,D). Similar results were obtained in three experiments with cells from three separate donors.

**Arecoline reduced production of IL-6, G-CSF, and GM-CSF at the protein level**

IL-6, G-CSF, and GM-CSF were found mainly in the culture supernatants, with comparatively low levels found in cell lysates, and arecoline significantly reduced production of these cytokines (P < 0.05) (Figs 2–4). Reduction relative to control cultures in cytokine production was seen by 239 μM arecoline (P < 0.05), while by 2150 μM and at 6440 μM arecoline, cytokine production was minimal (P < 0.05), reflecting cell death. Time course experiments revealed reduction in cytokine production relative to controls induced by arecoline by 8 h in most experiments performed (P < 0.05). Similar results were obtained in six dose response experiments with cells from six separate donors and in three time course experiments with cells from three separate donors.

**Arecoline reduced IL-1α and IL-1β in HUVEC**

Levels of IL-1α were similar in cell lysates as in culture supernatants (Fig. 5A,B), and reduced with increasing concentration of arecoline, with cytokine levels detectably reduced in both culture supernatants and lysates by 8.84 μM of arecoline (P < 0.05), and maximal reduction relative to controls seen by 2150 μM arecoline (P < 0.05). IL-1β was primarily in cell lysates and displayed a dose response more similar to that of IL-6, G-CSF, and GM-CSF, with appreciable reduction in lysate levels of IL-1β by 239 μM.
of arecoline ($P < 0.05$), and maximal reduction seen with 2150 μM arecoline ($P < 0.05$). Similar results were obtained in four experiments with cells obtained from four separate donors. Time course experiments showed minimal production of both these cytokines and a detectable amount was observed only after 24 h.

**Figure 1** Graphs showing the 24-h human umbilical vein endothelial cell (HUVEC) arecoline dose response (A, B) and time course (C, D) for basic fibroblast growth factor (bFGF) antigen levels in culture supernatants (B, D) and cell lysates (A, C). (A) Arecoline increased HUVEC lysate levels of bFGF in a dose-dependent manner, and this was maximal by 716 μM of arecoline ($P < 0.01$). (B) Although bFGF was primarily in cell lysates, lysate levels were greatly reduced by 2150 and 6440 μM with transfer of antigen to the supernatants due to cell death and lysis ($P < 0.01$). (C) Stimulation with 239 μM arecoline increased lysate bFGF levels by 4 h ($P < 0.05$), with little change on further incubation. (D) Significant reduction in cell-associated bFGF occurred by 8 h of treatment with 6440 μM arecoline ($P < 0.05$), with collection of the released bFGF in culture supernatants (D).

**Figure 2** Graphs showing the 24-h human umbilical vein endothelial cell (HUVEC) arecoline dose response (A, B) and time course (C, D) for IL-6 antigen levels in culture supernatants (B, D) and cell lysates (A, C). (A) Arecoline decreased HUVEC lysate levels of IL-6 in a dose-dependent manner, and this was maximal by 716 μM of arecoline ($P < 0.01$). (B) IL-6 was primarily in culture supernatants, and levels were greatly reduced by 239 μM arecoline ($P < 0.05$). (C) Stimulation with 239 μM arecoline reduced lysate IL-6 levels by 4 h ($P < 0.05$), and in supernatants by 12 h ($P < 0.05$) (D), while arecoline at 6440 μM achieved maximal reduction in IL-6 supernatant levels by 4 h incubation ($P < 0.05$).
Arecoline suppressed TNF-α stimulation production of IL-6 and GM-CSF

While Figs 3–5 demonstrate reduced background levels of inflammatory cytokines in endothelium treated with arecoline, we also sought to test the possibility that similar reduction occurs for stimulated cytokine production. Preliminary experiments demonstrated that the most pronounced cytokine synthetic response to TNF-α was in production of IL-6 and GM-CSF. Figure 6 demonstrates increased culture supernatant levels of IL-6 and GM-CSF.
upon treatment of HUVEC with TNF-α ($P < 0.05$) and suppression of this by arecoline to a similar extent to that seen in the absence of TNF-α ($P < 0.05$). Similar results were obtained in two experiments with HUVEC from separate donors.

Arecoline down-regulated IL-6, G-CSF, GM-CSF, IL-1α, and IL-1β mRNA expression by HUVEC but did not affect mRNA levels for bFGF

Figure 7 shows the results of three separate experiments with cells from three separate donors, comparing the relative amounts of mRNA detected by RT-PCR for all endothelial cytokines measured. Although arecoline did not significantly increase mRNA levels for bFGF, levels for IL-1α, IL-1β IL-6, G-CSF, and GM-CSF were all lower compared with control cells. The extent to which arecoline reduced mRNA levels differed among the cytokines, however, such that only very slight reduction was seen for IL-1α and the greatest reduction occurred in mRNA levels for GM-CSF.

Nicotine-induced bFGF antigen expression in HUVEC similar to arecoline, but acetylcholine agonists had no other similar effects on endothelial cytokine production compared with arecoline

To evaluate the possible action of arecoline via acetylcholine receptors in endothelium, HUVEC were treated with equimolar concentrations of arecoline, acetylcholine, nicotine, and muscarine. Figure 8 demonstrates a similar effect of nicotine to arecoline in increasing bFGF levels ($P < 0.05$), with arecoline being the more potent of the two agents with regard to increasing bFGF levels ($P < 0.01$). Neither acetylcholine nor muscarine had a similar effect. However, Fig. 9 demonstrates little if any effect of acetylcholine, nicotine, or muscarine on antigen

Figure 5 Graphs showing the 24-h human umbilical vein endothelial cell arecoline dose response for IL-1α (A, B) and IL-1β (C, D) in culture supernatants (B, D) and cell lysates (A, C). IL-1α was present in similar quantity in both cell lysates and supernatants (A, B), while IL-1β was primarily in cell lysates (C, D). Similar to the effect of arecoline on IL-6, G-CSF, and GM-CSF (Figures 2, 3, and 4), arecoline reduced levels of both IL-1α and IL-1β ($P < 0.05$). However, in the case of IL-1α reduction in cytokine levels were achieved at lower concentrations of arecoline than seen for other cytokines tested ($P < 0.05$).

Figure 6 Graphs of IL-6 and GM-CSF antigen in 24-h culture supernatants of human umbilical vein endothelial cells treated with or without TNF-α with increasing concentrations of arecoline. TNF-α increased both IL-6 and GM-CSF ($P < 0.05$), while arecoline reduced both TNF-α stimulated and background IL-6 and GM-CSF in a proportionately similar way ($P < 0.05$).
levels for IL-6, G-CSF, and GM-CSF. Similar results were obtained in three separate experiments with cells from three separate donors.

**Discussion**

Background expression of all factors studied varied greatly between experiments, but despite variability in the magnitude of responses, the overall pattern of response to arecoline was similar regardless of baseline expression, and this constant pattern of response independent of both magnitude of response and variable background is consistent with our earlier separate work studying cytokine expression by human endothelium and smooth muscle cells (42, 43, 50). Our observation that arecoline increases bFGF in cultured endothelium is consistent with elevated vascular bFGF in OSF detected by immunohistochemistry (29). As elevated bFGF and endothelial toxicity occurs at arecoline levels achieved during areca nut chewing (16, 24), the current data raise the possibility that habitual arecoline exposure may increase endothelial bFGF and that bFGF released by necrotic endothelium during local acute toxic episodes would contribute to OSF fibrosis. Ex-vivo experiments beyond the scope of the current study would be interesting to perform to further probe the possible role of endothelial bFGF in OSF.

The possibility that arecoline-induced endothelial bFGF contributes to OSF fibrosis is further consistent with the known activities of bFGF in both cell culture models and fibrotic disease (30–38, 41). Increased bFGF has been previously reported following injury of bovine aortic endothelium (51), and this supports one interpretation of our current data, such that increased HUVEC bFGF may represent a general endothelial response to maintain vascularity in sites of toxic injury. Because arecoline is a muscarinic stimulant, the absence of a clear similar response to acetylcholine or muscarine to that seen for arecoline supports a toxic as opposed to acetylcholine receptor-mediated mechanism for increased bFGF. The very modest effect of nicotine in increasing bFGF may be best explained in terms of a non-specific toxic effect, seen only at extremely high-nicotine concentrations. Notably, this observed response to nicotine also supports the concept of a ‘general raised bFGF response’ to injury. The absence of a signal peptide sequence in bFGF seems to also support the notion that bFGF is released upon plasma membrane disruption or cell death, for the purposes of restoring tissue integrity (52, 53).

Although we have interpreted endothelial bFGF release as potentially important for OSF fibrosis, it can equally well be argued that such release would help restore tissue vascularity via endothelial mitogenesis following endothelial necrosis. Related to this, while there was no evidence for arecoline-induced endothelial apoptosis in any of the current experiments described, or in our previous work (24), it is interesting that bFGF inhibits HUVEC apoptosis (54); endothelial apoptosis is induced upon bFGF withdrawal (55, 56); and apoptotic endothelium exerts negative feedback for further apoptosis via release of bFGF (42). On this basis, evolution may have selected for a comparable bFGF-mediated mechanism to both restore vascularity following toxic injury and to limit excessive endothelial apoptosis during tissue remodeling.

The reduced production of IL-1α, IL-1β, IL-6, G-CSF, and GM-CSF by HUVEC upon arecoline treatment appears
consistent with earlier studies demonstrating an anti-inflammatory potential in areca nut extracts (57, 58), while areca nut has been suggested as possessing significant analgesic and anti-inflammatory activity, potentially due to arecoline (59). Separately, however, there is up-regulation of some pro-inflammatory cytokines in OSF tissues (28, 60, 61), and OSF fibroblasts exhibit over expression of IL-6 induced by arecoline (62). These complex and seemingly opposing actions of areca nut and arecoline may reflect different capacities for response by differing cell types. In addition, areca nut contains many ingredients other than arecoline, which may possess inhibitory as well as stimulatory activity for these cytokines. It is acknowledged that the complex tissue response evidenced in OSF cannot be fully reflected in the isolated cell culture conditions employed in this study. Nonetheless, we do feel that our approach is reasonable, as it does permit generation of interpretable results, and has also provides basis for what seems a plausible model for endothelial bFGF in OSF fibrosis, amenable to further study for confirmation or refutation.

It is tempting to speculate that reduced endothelial inflammatory cytokine production upon arecoline exposure represents an adaptation for limiting potentially destructive inflammation in sites of toxic injury. Although a broad range of genes are reported as regulated in fibroblasts stimulated by arecoline (63), an adaptive anti-inflammatory endothelial arecoline response appears out of keeping with separate elevated IL-8 and RANTES in arecoline-treated bronchial epithelium (64). Arecoline activates the non-neuronal cholinergic pathway (65), which might contribute to anti-inflammatory activity and thus down-regulation of pro-inflammatory cytokines (66). However, in the current study, when HUVEC were treated with acetylcholine, muscarine, or nicotine, cytokine levels were not attenuated. Also, reduced IL-6 and GM-CSF production upon arecoline exposure in TNF-α treated HUVEC was proportionate to that seen in unstimulated cells, and this seems more consistent with a non-specific inhibitory effect of the toxin, than a receptor-mediated response. From this, reduced inflammatory cytokine levels in the current study most likely reflect indirect toxic activity of arecoline as opposed to a receptor-mediated processes. This conclusion is supported by detachment of ribosomes from the endoplasmic reticulum in arecoline-treated HUVEC, indicative of reduced overall mRNA and consequent protein production (24). The current observation of reduced mRNA for inflammatory cytokines is also supported by earlier observed reduction in ribosomal studding of endoplasmic reticulum (24).

References


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