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Alteration of gene expression profiles of peripheral mononuclear blood cells by tobacco smoke: implications for periodontal diseases

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Alterations of the host response by tobacco smoke adversely affect the periodontium. In this study, we examined the effects of *in vitro* acute smoke exposure on changes in m-RNA expression of primary peripheral mononuclear blood cells through microarray analysis. Mononuclear blood cells were isolated from four healthy non-smokers and plated in culture wells. Half of the cells were then exposed to 5 min of tobacco smoke. Fluorescent c-DNA probes were prepared from the linearly amplified m-RNAs for each sample and hybridized to cDNA microarrays representing ~30,000 human genes. Significant increases or decreases in m-RNA gene expression between non-smoke-exposed and smoke-exposed samples were identified by permutation *t*-test, as implemented by the Significance Analysis of Microarrays software package. After smoke exposure, the expression of 90 genes with known function was significantly elevated and the expression of 19 genes with known function was significantly depressed. In addition, 18 upregulated and 26 downregulated transcripts were expressed sequence tags with little information available on function. Approximately 20 of the significantly elevated genes had previously been reported in the literature to be associated with periodontal pathogenesis (fold changes in parentheses). These included plasminogen activator (4.4), Heat Shock Protein (Hsp) 40 kD (2.2), thrombomodulin (4.2), cytochrome c (1.8), COX-2 (2.6), interleukin-1a (1.4), chemokine ligand 1 (3.8), cathepsin L (2.0), and calgranulin A (2.1). In addition, several significantly elevated genes not previously reported in the literature may also play a role in periodontal pathogenesis, and thus warrant further investigation. These include Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor) (7.8), Hsp 10 kDa (1.7), Hsp 105 kD (2.1), Hsp 70 kDa (1.6), and mitogen activated protein kinase 3 (1.5). Among the significantly depressed genes that may play a protective or destructive role in periodontal pathogenesis were interferon gamma receptor 2 (0.58) and chemokine receptor 2 (0.24). Our results may be of use in the search for the molecular mechanisms for the adverse effects of tobacco smoke on the host response.

Key words: cytokines; lymphocytes; microarrays; monocytes; smoking

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Tobacco use is a major preventable risk factor in the incidence and/or progression of several diseases and conditions in the oral cavity. The most prominent of these include periodontal diseases and a variety of oral neoplasms (7, 17). Numerous studies have examined the effects of whole tobacco smoke as well as individual components of smoke on oral tissues such as

epithelium, connective tissue and components of the inflammatory and immune response. In the case of periodontal diseases, a variety of study methods including immunoassays and protein quantitation techniques have been used to assess changes in levels in inflammatory and host response substances resulting from tobacco use. While the general pattern emerging from previous studies is that tobacco smoke may induce an elevation of destructive inflammatory cell mediators and products such as interleukin (IL)-1β, Tumor Necrosis Factor α, and variety of metalloproteinases such as MMP-8 (2, 26, 28, 32), many potentially pathogenic products have not yet been explored.

Expression microarrays are a valuable new tool for examining the gene expression phenotype of any given sample. In this report, we identified several potentially pathogenic substances elevated as a result of tobacco exposure that could be targets for future investigation. Expression array analysis has been used recently to identify altered gene expression in macrophages exposed to bacterial pathogens (23). As this technique can measure the m-RNA/gene expression from the entire transcriptome (approximated by 30,000 candidate genes in this study), the task of deriving meaningful data and patterns can be quite daunting. Recently developed software tools such as the Significance Analysis of Microarrays (SAM) implement permutation *t*-test statistics which are helpful in identifying those genes whose expression is significantly elevated or depressed in different cell lines after acute smoke exposure (33). In this study we examined the effects of acute smoke exposure on the gene expression of mononuclear blood cells from medically healthy nonsmokers. Mononuclear blood cells may play a critical role in the host response for destructive periodontal diseases (24, 30). The SAM tool was used to identify a very small number (153/30,000 = 0.0051%) of significantly elevated or suppressed genes from the ~30,000 measured human genes in this study. The results and analysis from this microarray study confirm the general trends from previous studies on the effects of smoke on this mononuclear host response component in the oral cavity. Elevated gene expression is observed for proteins responsible for destructive inflammatory components of periodontal disease such as IL-1α, prostaglandins (prostaglandin synthase 2), and inhibitors of apoptosis (apoptosis inhibitor 2). In addition, several novel transcripts that may also play a role in periodontal diseases were modulated by smoke exposure in these studies. Thus, expression microarray analysis provides molecular targets which are candidates for modulators of the initiation and progression of periodontal diseases.

Material and methods

Isolation of peripheral mononuclear blood cells and smoke incubation

Peripheral blood mononuclear cells were isolated from four medically healthy nonsmokers (4 males, mean age 34.00 ± 13.24 SD). Subjects were included in the study if they did not take long-term anti-inflammatory drugs which could alter neutrophil function, and did not have a systemic condition such as diabetes or immunosuppression which could markedly alter mononuclear blood cell function. After obtaining informed consent, approximately 60 ml of peripheral blood was collected from each subject by venipuncture into heparinized tubes, diluted 1 : 1 in phosphate buffered saline (PBS), layered over Histopaque 1077 (Sigma Diagnostics, St. Louis, MO), and centrifuged for 10 min at 700 × *g* to separate out the monocyte/lymphocyte fraction from granulocytes and erythrocytes. The monocyte/lymphocyte layer was drawn off into PBS, centrifuged to isolate the cell pellet and treated briefly with distilled water to lyse any residual erythrocytes in the preparation. The cells were then resuspended in RPMI 1640 media with 25 mM HEPES and adjusted to a concentration of 4×10^6 cells/ml via a hemocytometer. Aliquots 1 ml of each cell suspension were then plated into chambers of 24-well culture dishes and incubated for 90 min at 37°C in a 5% CO_2 humidified atmosphere to promote adherence of mononuclear blood cells to the bottom of the well. Non-adherent cells were then removed by aspiration, and the wells were washed three times with PBS. RPMI 1 ml 1640 media with 25 mM HEPES was then added to each well.

The adherent mononuclear blood cells in culture wells were then exposed to *in vitro* smoke in a system previously described (28, 29) for 5 min or not exposed to further *in vitro* smoke (baseline controls). The culture dishes were then incubated for one additional hour without further smoke exposure at 37°C in a 5% CO_2 humidified atmosphere. Immediately following this incubation, the adherent cell wells in the culture well were frozen and stored in a –80°C freezer. Within 2 days the frozen cells were transported on dry ice to the UCSF Cancer Center for RNA extraction.

RNA preparation and microarray protocols

Total RNA from mononuclear blood cells was isolated with the Qiagen RNeasy Mini kit (Qiagen Inc., Valencia, CA). mRNA in 500 ng of total RNA was amplified via the T7 strategy as previously described (3), with ethanol precipitation steps replaced by purification on Zymo DNA clean and concentrator-5 columns (Zymo Research, Orange, CA). Amplified RNA was converted to amino-allyl modified cDNA and coupled to N-hydroxysuccinimidyl esters of fluorescent Cy3 or Cy5 (Amersham-Pharmacia, Inc., Piscataway, NJ) using a protocol first developed at Rosetta Inpharmatics (14).

Arrays were done in the Haqq laboratory at UCSF using standard methods (www.microarrays.org). DNA was prepared by colony PCR (4) of the research genetics sequence verified human clone set, plates 1 to 417 (Research Genetics, Huntsville, AL). The cDNAs were amplified by using M13 forward and reverse primers in a 100-l PCR with 0.2 M final concentration (each) of forward C082, 5′-CGCCAGGGTTTT-CCCAGTCACGA-3′ and reverse C083, 5′-AGCGGATAACAATTTCACACAG-GA-3′ primers, 250 M dNTPs, and 1 unit of Taq in 1 × PCR buffer. Amplified cDNA was then spotted onto poly (L-lysine) coated glass slides using a custom built arrayer with 32 tips (SMP3, TeleChem International, Sunnyvale, CA). Arrays were post processed to minimize the background caused by oxidation of the polylysine coating by the ''shampoo method'' suggested by Joe DeRisi, Department of Biochemistry, UCSF. In this technique, slides were placed in 3 × SSC, 0.2% SDS for 5 min at 65°C, rinsed in water, then 95% ethanol, and spun dry. Subsequent post-processing steps used succinic anhydride as described at www.microarrays.org.

Fluorescent probes were applied to microarrays under lifterslips (Eerie Scientific) and hybridized for 14–16 h at 63.5°C in a HybChamber (GeneMachines, San Carlos, CA). After hybridization, slides were washed in 2 × SSC/0.1% SDS warmed to 65°C until the coverslips dropped off. Further room temperature washes were performed with 2 × SSC gently shaking for 4 min, and then in 0.065 × SC for 1 min. After washing, slides were spun dry. Microarrays were scanned on an Axon Genepix4000B confocal laser scanner, and primary data analysis was done using Genepix version 3.0 software (Axon Instruments, Foster City, CA).

Data analysis

Gene expression was visualized with cluster and Java tree view (http://genomewww5.stanford.edu/~alok/TreeView/download/index.html). Genepix Median of Ratio values from duplicate experiments were filtered for genes where data was available in 80% of experiments, and filtered for at minimum one experimental value showing minimum twofold absolute value in ratio. Of 41,000 loci, 9870 passed these filtering criteria. For SAM, the filtered cluster data table containing data these 9870 loci was used as the input for this permutation *t*-test algorithm (32). Output from SAM analyses was visualized by Microsoft Excel macros in converter version 2.0 obtained from Tony Dobson (Department of Obstetrics and Gynecology, UCSF).

To determine what is currently known regarding the ontologies (family of function) and specific functions of significantly elevated or suppressed genes in this study, the gene accession numbers for each significant gene were cross-referenced with the gene data bank at the Stanford Online Universal Resource for Clones and ESTs (SOURCE; source.stanford.edu/cgi-bin/sourceSearch). This online resource supplies information for known genes including gene ontologies and summaries of known gene functions with PubMed® links to published literature on the selected gene. The summary function tables presented in the Results section are derived from information from SOURCE databases.

Results

The SAM software tool to implement permutation *t*-test analysis, which takes into account the statistical problem of multiple comparisons (thousands of assays are performed on four samples), demonstrated significantly elevated or depressed m-RNA expression after exposure of *in vitro* smoke. Using this significance screening criteria (at this delta value of 0.76, SAM estimates a false discovery rate of zero), only a small fraction of the ~30,000 tested gene loci demonstrated a significant change in m-RNA expression (0.0037% = 155/30,000). Specifically, 108 genes showed significant elevation and 45 genes significant depression of m-RNA expression. Of these 108 significantly elevated genes, 90 have been named with some data on function as of the time of submission of this manuscript. Of the 45 depressed genes, 19 have been named. The remaining 18 upregulated and 26 downregulated transcripts are expressed sequence tags (ESTs) with little information available on function.

Table 1 summarizes the 90 identified elevated genes with known function after smoke exposure from the mononuclear blood cells from the four nonsmoking subjects. Each of the identified genes is listed by the gene name, gene bank accession number, relevant ontologies to periodontal disease as derived from the SOURCE gene data bank (ontology is the classification of function in which the gene product may play a role), and other relevant descriptions of the gene function as derived from the SOURCE gene data bank. The mean expression values (viz. the relative intensity of staining from the fluorescent probe) for the subjects for each gene before and after smoke exposure, as well as the ratio of these means are given for each gene. For the purposes of the SAM analysis, a ''d'' value is also given which represents the ''ratio'' value divided by the standard deviation between the samples (corrected with a constant value) (32). This calculation is similar to other statistical approaches to calculate the ''normalized effect'', using the mean of the change divided by the standard deviation. For the purposes of presentation, gene products implicated in the pathogenesis of periodontal diseases in either a protective or a destructive role that have been described in the past literature are highlighted in yellow in Tables 1 and 2. Those genes or gene products that have been implicated to play a general destructive and or protective role in other inflammatory responses in the body, but not yet reported for periodontal diseases, are highlighted in orange. Finally, those genes that have been implicated in the general cell activation and signaling pathways are highlighted in green. The determinations of this color-coding classification were made by cross-referencing the gene product with the key words ''periodontitis'' and ''periodontal diseases'' using the PubMed® link to the SOURCE web site.

Among the previously reported elevated genes implicated in the published literature to date for the destructive inflammatory response for periodontal diseases were a variety of cytokines, chemokines, and inflammatory substances. These genes included those for plasminogen activator, thrombomodulin, several heat shock proteins (Hsp's), prostaglandin synthases, IL-1α, cathepsin L, enzymes associated with the oxidative burst (cytochrome c and NADH dehydrogenase) and with bone homeostasis (calgranulin A and calcitonin receptor modifying protein). In addition, there were several significantly elevated genes not previously reported in the literature that may also play a role in periodontal pathogenesis. These include Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor), Hsp 10 kDa, Hsp 105 kD, Hsp 70 kDa, and mitogen activated protein kinase 3.

Table 2 summarizes the 19 identified significantly depressed genes after smoke exposure from the mononuclear blood cells

Notes for Tables 1 and 2

Each of the Significantly Elevated Genes (Table 1) or Significantly Depressed Genes (Table 2) is identified by gene name (first column) and gene bank accession number (second column). In the third column, the relevant ontologies of the gene to periodontal disease are presented in italicized capital letters, followed by other relevant descriptions of the gene function (as derived from the SOURCE gene data bank). In the fourth and fifth column, the mean expression values among the subjects (viz. the relative intensity of staining from the fluorescent probe) for each gene before and after smoke exposure are given. In the sixth column, the ratio of these means are given for each gene are presented. This ratio represents the magnitude of the elevation or depression of gene expression after smoke exposure. In the seventh and last column, a ''d'' value is given which represents the ''ratio'' value from the sixth column divided by the standard deviation between the samples (corrected with a constant value) (33). This calculation is similar to other statistical approaches to calculate the ''normalized effect", using the mean of the change divided by the standard deviation. Those gene products that have been implicated in the pathogenesis of periodontal diseases in a protective and/or destructive role, and that have been described in the past literature, are highlighted in yellow in these tables. Those genes or gene products that have been implicated as playing a general destructive and or protective role in other inflammatory responses in the body, but not yet reported for periodontal diseases, are highlighted in orange. Finally, those genes that have been implicated in the general cell activation and signaling pathways are highlighted in green. The determinations of this color-coding classification were made by cross-referencing the gene product with the key words ''periodontitis'' and ''periodontal diseases'' using the PubMed® link to the SOURCE web site.

Table 1. Significantly elevated genes

Gene Name	Gene Bank Accession Number	*ONTOLOGIES*/SUMMARY FUNCTION	Mean smoke-exposed gene expression	Mean non smoke-exposed gene expression	RATIO	''d'' value
Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	R14663	*POSITIVE REGULATION OF CELL PROLIFERATION, PATHOGENESIS*/ may be involved in macrophage-mediated cellular proliferation. It is mitogenic for fibroblasts and smooth muscle but not endothelial cells	36938	4755	7.77	81.746
Plasminogen activator, tissue	AA453728	*PROTEOLYSIS*/Tissue-type plasminogen activator; serine protease that converts inactive plasminogen to plasmin. This enzyme plays a role in cell migration and tissue remodeling	21813	4947.75	4.41	59.564
Hypothetical protein DJ159A19.3	T60121		2296.5	1267	1.81	22.170
Cullin 1	AA486790	*PROTEOLYSIS*/ may target other proteins for ubiquitin-dependent proteolysis	40249.75	4969.75	8.10	21.049
FBJ murine osteosarcoma viral oncogene homolog B	T62179	*REGULATION OF CELL CYCLE*/ member of the FOS family	28919.75	3801.5	7.61	20.823
Activating transcription factor 3	H21041	*REGULATION OF TRANSCRIPTION*	12597.5	3554.25	3.54	20.763
Kruppel-like factor 4 (gut)	H45711	*TRANSCRIPTION FACTOR ACTIVITY*/ Endothelial Kruppel-like zinc-finger protein 4; regulates genes involved in epidermal barrier function	11612.5	5276.75	2.20	12.508
Amphiregulin (Schwannoma-derived growth factor)	AA857163	*CYTOKINE ACTIVITY, GROWTH FACTOR ACTIVITY*/ inhibits the growth of certain carcinoma cell lines but stimulates the growth of fibroblasts and epithelial cells	3385.5	1044	3.24	11.729
Homo sapiens, clone IMAGE:5754422, mRNA	H56345		2837.25	1296.75	2.19	11.234
Hypothetical protein FLJ32499	W56590		5335.5	1871.25	2.85	11.198
ATPase, H+ transporting, lysosomal 16 kDa, V0 subunit c	AA486138	*PROTON TRANSPORT*/part of the proton channel	28051.75	14634.25	1.92	11.076
Jagged 1 (Alagille syndrome)	R70685		2421.75	1370.75	1.77	11.069
DnaJ (Hsp40) homolog, subfamily B, member 1	AA435948	*HEAT SHOCK PROTEIN*/Member 1 of the DNAJ/HSP40 family of heat shock proteins; may prevent aggregation of newly translated proteins	5203	2390.5	2.18	10.584
Suppressor of cytokine signaling 3	T72915	Negative regulator in the JAK-STAT pathway	6893.5	2238.5	3.08	9.822
Pleckstrin homology-like domain, family A, member 1	AA258396	*APOPTOSIS*/may promote apoptosis	37611	19043.5	1.98	9.472
Cytochrome c	AA865265	*INDUCTION OF APOPTOSIS, ELECTRON TRANSPORT*	3883	2141.5	1.81	9.180

Thrombomodulin precursor	AA256378	*BLOOD COAGULATION*/may play a role in cardiovascular diseases	2253.5	535.5	4.21	9.154
Enhancer of polycomb homolog 1	W93523		8081.75	5118.5	1.58	4.920
Thrombomodulin	H59861	*BLOOD COAGULATION*/may play a role in cardiovascular diseases	7298.5	1698.25	4.30	8.792
V-maf musculoaponeurotic fibrosarcoma oncogene homolog B	T50121	*TRANSCRIPTION FACTOR*	30417	10690	2.85	8.779
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	AA644211	*INFLAMMATORY RESPONSE/* cyclooxygenase 2: regulates angiogenesis and cell migration, catalyzes the rate-limiting step in the formation of inflammatory prostaglandins	2633.5	1003	2.63	6.707
V-jun sarcoma virus 17 oncogene homolog (avian)	W96134	*REGULATION OF TRANSCRIPTION AND CELL GROWTH*/Proto-oncoprotein	15238.25	4917.5	3.10	7.614
Topoisomerase-related function protein 4-2	AA447753		6361	3726.5	1.71	8.148
V-jun sarcoma virus 17 oncogene homolog (avian)	W96134	*REGULATION OF TRANSCRIPTION AND CELL GROWTH*/Proto-oncoprotein	15238.25	4917.5	3.10	7.614
Homo sapiens clone 23822 mRNA sequence	R39546		1251.25	696.75	1.80	7.193
Interleukin 1α	AA936768	*INFLAMMATORY RESPONSE, CHEMOTAXIS, CELL PROLIFERATION*	1026.75	725	1.42	7.172
Snail homolog 1 (Drosophila)	AA464983	may act as a transcriptional repressor; contains a zinc-finger DNA binding domain	3611.75	1779.5	2.03	7.120
Partner for ARF 1	R40324		10889.5	6002	1.81	7.113
Glutathione S-transferase M1	AA290737	*TUMOR SUPPRESSOR*/Member of the mu class of glutathione S-transferases	26584.5	11274	2.36	7.021
Splicing factor, arginine/serine-rich 3	AA598400	*MRNA SPLICING*	41805.25	22658	1.85	6.966
Plakophilin 2	H66158	*CELL-CELL ADHESION*	4412.25	2741.25	1.61	6.901
DnaJ (Hsp40) homolog, subfamily A, member 1	AA490946	*HEAT SHOCK PROTEIN*/Significantly homologous to bacterial heat shock protein DNAJ; may function in protein folding and transport	13063.5	6371.75	2.05	6.860
Gardner–Rasheed feline sarcoma viral (v-fgr) oncogene homolog	AA256231	*TYROSINE KINASE ACTIVITY*	33653.75	13159.75	2.56	6.693
Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	W46900	*INFLAMMATORY RESPONSE, CHEMOTAXIS*/has chemotactic activity for neutrophils.	14017.25	3729.75	3.76	6.313
Serine (or cysteine) proteinase inhibitor, clade I (neuroserpin), member 1	AA115876	*PROTEASE INHIBITOR*/serine protease inhibitor that inhibits plasminogen activators and plasmin but not thrombin	2780.75	1767	1.57	6.439
Homo sapiens cDNA FLJ20528 fis, clone KAT10770	H96235		6590	4044.75	1.63	6.322
Homo sapiens cDNA FLJ33107 fis, clone TRACH2000959	AA427406		12679.5	6402.5	1.98	6.282

Table 1. continued

Gene Name	Gene Bank Accession Number	*ONTOLOGIES*/SUMMARY FUNCTION	Mean smoke-exposed gene expression	Mean non smoke-exposed gene expression	RATIO	''d'' value
Cathepsin L	W73874	*PROTEOLYSIS*/important for the overall degradation of proteins in lysosomes	24360.25	11932.75	2.04	6.053
Dual specificity phosphatase 5	W65461	*PHOSPHATASE ACTIVITY*/displays phosphatase activity toward several substrates. The highest relative activity is toward erk1.	8676	4470.25	1.94	5.985
Homo sapiens mRNA; cDNA DKFZp434O1311 (from clone DKFZp434O1311)	W93482		2514.25	1602	1.57	5.921
Thyroid receptor interacting protein 8	AA425650	specifically interacts with the ligand binding domain of the thyroid receptor	585.25	444.25	1.32	5.606
Isopentenyl-diphosphate delta isomerase	H08899	*CHOLESTEROL BIOSYSNTHESIS*	12055.25	7470.25	1.61	5.506
Conserved gene telomeric to alpha globin cluster	H77729	Widely expressed protein	3727.25	2804.75	1.33	5.430
Phospholipase A2, group IIA (platelets, synovial fluid)	T61323	*ONCOGENESIS, LIPID CATABOLISM*/ thought to participate in the regulation of the phospholipid metabolism in biomembranes including eicosanoid biosynthesis.	1807.5	1306.75	1.38	5.430
Homo sapiens cDNA FLJ37247 fis, clone BRAMY2006397	N36923		4914	3038	1.62	5.370
Heat shock 10 kDa protein 1 (chaperonin 10)	AA448396	*HEAT SHOCK PROTEIN*/Chaperonin 10; interacts with chaperonin 60 (HSPD1) to refold denatured proteins	7344.5	4573	1.61	5.356
Thiamin pyrophosphokinase 1	W84815	a cellular enzyme involved in the regulation of thiamine metabolism	3418.5	2014.5	1.70	5.278
Misshapen/NIK-related kinase	R02058	*REASPONSE TO STRESS, PROTEIN KINASE CASCADE*/encodes a serine/threonine kinase	2180	1557.75	1.40	5.269
Solute carrier family 2 (facilitated glucose transporter), member 3	T97889	*GLUCOSE METABOLISM*/facilitative glucose transporter. probably a neuronal glucose transporter	15174.5	5787.25	2.62	4.717
Heat shock 105 kD	AA485036	*HEAT SHOCK PROTEIN* member of the high molecular weight family of heat shock proteins	28115.75	13478.25	2.09	5.234
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5 kDa	AA111999	*ENERGY PATHWAYS*/transfer of electrons from NADH to the respiratory chain	11490	7592.5	1.51	5.101
Eukaryotic translation initiation factor 5	H40023	*REGULATION OF TRANSLATION*	2575	1473.25	1.75	4.963
Enhancer of polycomb homolog 1	W93523		8081.75	5118.5	1.58	4.920
Dehydrogenase/reductase (SDR family) member 4 like 2	AA429946		6858.5	4809.25	1.43	4.892

Nuclear receptor subfamily 4, group A, member 1	N94487	*SIGNAL TRANSDUCTION/*This gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. Translocation of the protein from the nucleus to mitochondria induces apoptosis.	6466.25	4218	1.53	4.891
Mitogen-activated protein kinase kinase 3	H08749	*SIGNAL TRANSDUCTION/* phosphorylates and activates the MAP kinase p38 (CSBP1)	3780.75	2586	1.46	4.836
Mitochondrial translational initiation factor 2	H18070	*REGULATION OF TRANSLATION*	3043.25	2247.75	1.35	4.823
Hypothetical protein BC009331	T49222		24029	8806.25	2.73	4.805
NEFA-interacting nuclear protein NIP30	T62832		972.5	692.5	1.40	4.782
Receptor (calcitonin) activity modifying protein 1	AA133215	*CALCIUM TRANSPORT/*transports calcitonin-receptor-like receptor to the plasma membrane where it acts as a calcitonin-gene-related peptide receptor.	3123.25	2321.75	1.35	4.775
Chromosome 6 open reading frame 16	AA127234	may be associated with psoriasis vulgaris	825.25	480	1.72	4.771
Serine threonine kinase 39 (STE20/SPS1 homolog, yeast)	H84871	*RESPONSE TO STRESS/*Function may act as a mediator of stress-activated signals	5151.25	3020	1.71	4.771
Heat shock 70 kDa protein 2	AA455102	*HEAT SHOCK PROTEIN/* member of the heat shock HSP70 family of molecular chaperones	2986.75	1853.75	1.61	4.769
Likely ortholog of mouse deleted in polyposis 1	H99681		2086.75	1523.25	1.37	4.743
Solute carrier family 2 (facilitated glucose transporter), member 3	T97889	*GLUCOSE METABOLISM/*Facilitated glucose transporter	15174.5	5787.25	2.62	4.717
Retinoblastoma binding protein 6	R88741	*PROTEIN BINDING*	10241.75	6294	1.63	4.685
Transactivated by hepatitis C virus core protein 1	AA137196		1782.25	1096.75	1.63	4.684
GK001 protein	AA004671		746.75	415.75	1.80	4.662
Proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)	T75041	*NERVE ENSHEATHMENT/*plays an important role in the formation or maintenance of the multilamellar structure of myelin.	35521	21803.25	1.63	4.652
Homo sapiens cDNA FLJ31636 fis, clone NT2RI2003481	H14816		2401.5	1842	1.30	4.609
Ras-related associated with diabetes	W84445		1270.5	786.5	1.62	4.609
Protein tyrosine phosphatase type IVA, member 1	R61674	*ONCOGENESIS*	19188.75	11119.5	1.73	4.576
Ornithine decarboxylase antizyme inhibitor	AA676515	*POLYAMINE SYNTHESIS/*Antizyme inhibitor; binds to and releases ornithine decarboxylase and thereby inhibits its degradation	3494.5	2393	1.46	4.567
Myeloid leukemia factor 2	AA480835		20727.25	12603	1.64	4.502
PHD protein Jade-1	AA136664		6185.25	4050.75	1.53	4.493

Table 1. continued

Gene Name	Gene Bank Accession Number	*ONTOLOGIES*/SUMMARY FUNCTION	Mean smoke-exposed gene expression	Mean non smoke-exposed gene expression	RATIO	''d'' value
Periphilin 1	T71365		18485.25	7971.25	2.32	4.481
WAS protein family, member 3	AA629542	*ACTIN CYTOSKELETON*/nucleates actin polymerization	3575	2856.75	1.25	4.430
S100 calcium binding protein A8 (calgranulin A)	AA086471	*INFLAMMATORY RESPONSE*/expressed by macrophages in chronic inflammations	4504.5	2158.25	2.09	4.421
Ribosomal protein L21	AA464034	*PROTEIN BIOSYNTHESIS*	35125	19326.5	1.82	4.408
Ubiquitin specific protease 7 (herpes virus-associated)	AA064681	*VIRAL PATHOGENESIS*/cleaves ubiquitin fusion protein substrates. binds to the herpes virus protein vmw110 which may therefore modulate its substrate specificity or activity to stabilize viral proteins	4517	2212	2.04	4.406
Nucleolar GTPase	AA446682		12202	7628	1.60	4.369
SRY (sex determining region Y)-box 2	AA451892	*MAINTENANCE OF CHROMATIN ARCHITECTURE*	1054.25	708	1.49	4.319
Nescient helix loop helix 1	H09936	may have a role in development of the nervous system	1171.75	936.5	1.25	4.310
Phospholipase C-like 1	AA411387	*INTRACELLULAR SIGNALING, LIPID METABOLISM* initiates the inositol phospholipid signaling pathway	1373.75	935.25	1.47	4.308
Mal, T-cell differentiation protein	AA227594	may be involved in membrane signaling in activated T cells	6085	3739.5	1.63	4.283
Bromodomain adjacent to zinc finger domain, 2A	W88615		6158	4844.75	1.27	4.256
Runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	AA146826	*ONCOGENESIS/* hematopoietic transcription factor	1646.5	692.5	2.38	4.231
ALL1-fused gene from chromosome 1q	AA456008	*CELL GROWTH, MAINTENANCE*	1520.75	1251.5	1.22	4.226
Adenylate cyclase 7	AA463976	converts ATP to cAMP	2110.25	1481.5	1.42	4.210
Phosphatidylserine receptor	AA459945		1357.5	728.25	1.86	4.203

Table 2. Significantly depressed genes

GENE NAME	Gene Bank Accession Number	SUMMARY FUNCTION	Mean smoke-exposed gene expression	Mean non smoke-exposed gene expression	RATIO	''d'' value
Chemokine (C-C motif) receptor 2	H58254	*CELLULAR DEFENSE RESPONSE, INVASIVE GROWTH, INFLAMMATORY RESPONSE, CHEMOTAXIS*/encodes two isoforms of a receptor for monocyte chemoattractant protein-1, a chemokine which specifically mediates monocyte chemotaxis. Monocyte chemoattractant protein-1 is involved in monocyte infiltration in inflammatory diseases.	2353	9962	0.24	0.0018
Nudix (nucleoside diphosphate linked moi)	N33851	*CELL-CELL SIGNALING*	242.25	402	0.60	0.0689
Putative serine-threonine protein kinase	AA463188	may play a role in regulating the mitotic cycle	3225	5946.25	0.54	0.0695
Protein kinase C binding protein 2	H50086	*CELL GROWTH AND MAINTENANCE*	1128.5	1904.5	0.59	0.1052
Homo sapiens clone 24422 mRNA sequence	AA599072		243	353.25	0.69	0.1096
Lipoma HMGIC fusion partner-like 2	AA863469	Low similarity to LHFP	2582.25	5118	0.50	0.1349
Phosphatase and tensin homolog (mutated)	W37864	potential tumor suppressor	2872	5387.25	0.53	0.1583
KIAA0172 protein	AA464605		1785.25	3187	0.56	0.1640
Homo sapiens zinc-fingers and homeoboxes	N50828	*REGULATION OF TRANSCRIPTION*	760.75	1034.5	0.74	0.1647
Sema domain, seven thrombospondin repeat	AA436152	may act as positive axonal guidance cues	1279.5	2111.25	0.61	0.1660
Suppressin (nuclear deformed epidermal a	AA425806	*REGULATION OF TRANSCRIPTION*	4416.25	8345.75	0.53	0.1703
Carboxylesterase 1 (monocyte/macrophage)	AA043436	RESPONSE TO TOXIN/Monocyte/macrophage serine esterase; involved in drug detoxification	272.5	292.25	0.93	0.1827
Apoptosis inhibitor 2	H48706	*APOPTOSIS/* may bind to TRAF1 and TRAF preventing activation of ICE-like proteases	5461.25	9769	0.56	0.1871
LIM domain only 2 (rhombotin-like 1)	AA464644	*ONCOGENESIS, CELL GROWTH AND/OR DEVELOPMENT*/seems to be critical for the regulation of red blood cell development.	4155.5	10641.75	0.39	0.1880
Galactose-1-phosphate uridylyltransferas	AA857212	*GALACTOSE METABOLISM*	757.5	1227	0.62	0.1922
Interferon gamma receptor 2 (interferon)	AA448929	*RESISTANCE TO PATHOGENIC BACTERIA, RESPONSE TO VIRUSES*	8595	14848.5	0.58	0.1998
Myosin phosphatase, target subunit 1	AA669126	regulates the interaction of actin and myosin	619.5	739.25	0.84	0.2013
BAI1-associated protein 2	H46962	*INSULIN RECEPTOR SIGNALING PATHWAY, AXONOGENESIS*	1293.25	1591.5	0.81	0.2017
RAN binding protein 6	AA489790	may be involved in the nuclear import of repeat-containing ribosomal proteins	3473.25	5176.5	0.67	0.2033

of the four nonsmoking subjects. The most significantly depressed of these genes is chemokine receptor 2, which is responsible for recruitment of monocytes into sites of inflammation. Other suppressed genes in this list that have been associated with the inflammatory/immune response included interferon (IFN)-γ receptor 2.

Discussion

As of this writing, this is the first report of the use of microarrays to examine the effects of whole tobacco smoke on gene expression of either human cells or human tissues. The only related microarray study on effects of tobacco products on tissues was a study that examined the effects of benzo (a)pyrene (a component of tobacco smoke) on murine vascular smooth muscle cells (16). In that study the investigators noted changes in gene expression of lymphocyte antigen-6 complex genes, histocompatibility class I component factors, secreted phosphoproteins and several interferon inducible proteins. The authors concluded that these findings could open ''new venues of exploration'' in the understanding of the molecular basis of atherogenesis from oxidative stress (16). Indeed, the use of microarrays for analysis of gene expression of cells before and after treatment with tobacco smoke is a technique with considerable potential. However, the measurement of ~30,000 genomes from cells from few subjects or cell lines presents a statistical challenge, since some associations with treatment are expected to occur by chance.

To overcome the statistical limitation of multiple tests in few samples, three approaches have been used in the microarray literature. First, some studies have defined modulated transcripts in a test set, and then used an independent dataset to confirm results (13). Other investigators have permuted expression array datasets leaving one sample out with each iteration and assessing modulated transcripts in remaining samples. Additional permutation tests scramble the labels of array samples (e.g. smoke exposed vs. non-smoke exposed) and ask how many times an association with treatment is found for a particular gene in the scrambled set as compared to the original data. This type of permutation test allows a convenient estimate of the false discovery rate, which is simply the percentage of times an association is found in scrambled datasets. We chose to use SAM, which implements permutation analysis under very strict conditions where the algorithm predicted 13% false identification of smoke-regulated genes. Thus, our results are conservative and likely represent an underestimation of the true number of transcripts modulated by smoke in primary mononuclear cells. The SAM approach is one attempt to distill meaningful results from such a large gene data base from a small number of subjects (33).

Keeping these potentials and limitations of the microarray approach in mind, the observations of this study generally confirm previous work which has focused on the role of tobacco smoke in altering the mononuclear response in periodontal diseases. While this study focused on genes implicated in periodontal diseases, a similar analysis can be done for those genes implicated in the initiation and progression of oral neoplasms. This microarray analysis revealed a significant elevation of several previously unreported candidate genes for the pathogenesis of periodontal diseases.

Among the inflammatory substances, significant elevated expression of mRNA's was observed for several of the inflammatory cytokines responsible for the progression of periodontal disease. These include genes for IL-1α, cyclooxygenase-2, phospholipase A2 and NADH dehydrogenase. IL-1α (together with IL-1β) may have multiple adverse effects on periodontal tissues including increased bone resorption, increased collagen turnover, and stimulation of other inflammatory cytokines (8). Cyclooxygenase-2 coupled with phospholipase A2 are critical pathway enzymes for the synthesis of prostaglandins in inflammation (24). NADH dehydrogenase and cytochrome c are enzymes in the oxidative burst pathway of inflammatory cells (2, 28). In addition, other elevated genes in smoke-treated mononuclear blood cells that have been implicated in the destructive inflammatory processes of periodontal diseases included several heat shock proteins (15, 35), cathepsin L (10), plasminogen activator and thrombomodulin (20, 22), as well as Mitogen Activated Protein protein kinase (1, 31). In addition, several genes responsible for calcium homeostasis which have been implicated in the destructive and reparative pathways of periodontal diseases were also elevated. These included the S100 calcium binding proteins (9, 21), calgranulin, and calcitonin receptor. Furthermore, there was a suppression of several genes that may have a role in protecting the host and/or suppressing the destructive effects of the periodontal inflammatory response. These include IFNα and chemokine receptor 2 (11, 18). It is interesting to note that among the elevated and suppressed genes in smoke-treated mononuclear cells, the general pattern was that genes responsible for the more destructive effects of inflammation appeared elevated, while genes responsible for more regulatory effects appeared suppressed.

However, the elevation and suppression of certain genes may be a protective or compensatory response to the potential destructive and/or toxic effects of smoke exposure. In addition, some gene products may have both protective and destructive effects on the periodontium. For example the gene for glutathione S-transferase M1 was significantly elevated. This enzyme is responsible for protection of cells against toxic substances (2, 15). Elevation of this gene may therefore be a protective response to exposure to the toxic substances in smoke. However, products of glutathione metabolism may enhance the growth and virulence of periodontopathic bacteria such as *Treponema denticola* (6). The genes for several protease inhibitors (e.g. serine protease inhibitor) were also slightly elevated. These protease inhibitors may also have a protective effect against smoke-stimulated proteolytic enzyme activities (25).

Several other genes were significantly elevated which, although not previously described in the periodontal research literature, may also play a role in tobacco-related pathogenesis of periodontal diseases. The most significantly elevated of these genes was the diphtheria toxin receptor with ~7.8-fold elevation when exposed to tobacco smoke. This gene is a heparin-binding epidermal growth factor-like growth factor. It has been implicated in macrophage mediated cellular proliferation (23), is mitogenic for fibroblasts, modulates neutrophil function (34), and has been implicated in the development of atherosclerosis (27). Other significantly elevated genes in this study that have been indirectly implicated in periodontal pathogenesis include the neutrophil chemoattactant chemokine (C-X-C motif) ligand 1 (18).

In summary, the use of amplified microarray analysis of primary mononuclear cell gene expression is a useful tool for confirming or refuting a variety of previous studies which have suggested how tobacco smoke alters the host/inflammatory response in periodontal diseases. Investigation into the role of gene products not previously recognized to be modulated by smoke, such as diphtheria toxin receptor, and heat shock proteins as mediators of smoke-induced damage are warranted. Such studies may include modulation of

expression of these genes in experimental systems with investigation of the consequences to inflammation, bone resorption, and other features of periodontal disease.

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