

Stress and Atherogenesis: Smooth Muscle Cell Mitogenic Activity and other Biochemical Changes Associated with Sera of "Stressed" Subjects

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ABSTRACT

The proliferation of smooth muscle cells in the arterial wall (VSMC) is considered to play a key role in the development of atherosclerosis. To investigate the possible contribution of "stress" (experimentally-induced) to this process, blood from healthy volunteers, ages 21 to 65, screened to exclude major risk factors for coronary heart disease, was assayed for mitogenic activity after the subjects were exposed to one of 2 "stress" conditions. These consisted of a cognitive task with superimposed verbal harassment (group 1), and the cognitive task without harassment (group 2). Mitogenic activity was determined by studying the growth stimulatory effects of PDGF-depleted plasma derived serum (PDS) from "stressed" subjects added to cultured VSMC, as measured by incorporation of radioactive thymidine into DNA or increase in cell number. In addition, changes in the steady state of the mRNA for the c-myc protooncogene were also assayed in VSMC by Northern blot analysis, using sera showing the greatest differential "pre/post stress" mitogenic activity. Blood pressure (BP), heart rate (HR), cortisol, and serum total and HDL cholesterol were also evaluated. All measurements were made immediately before (baseline) and after a 30 min interval. Analysis of the data revealed that there were 33% of subjects in group 1 with an increase of thymidine incorporation 15% or greater than baseline, versus 21% in group 2. The average increases were 45% and 30%. A higher percentage (35-42%) of subjects in group 1 responded with increases in systolic and diastolic blood pressure, compared to subjects in group 2 (15-20%); the average in blood pressure was 10-15%. Similarly, more subjects (52%) in group 1 had an elevated (average 10-15%) serum cortisol, compared to the 42% in group 2 subjects. HR, total HDL cholesterol showed slight changes only. These results suggest that psychoactive factors may affect cardiovascular systems via rapid elicited rises in serum mitogenic activity for VSMC. *Iran. Biomed. J* 2: 59-70, 1998.

Keywords: Stress, atherogenesis, smooth muscle cell proliferation

INTRODUCTION

Over the last several decades, significant progress have been made in identifying risk factors for coronary heart disease (CHD), still considered the leading cause of morbidity and mortality in many countries. Some 200 coronary risk factors (CRF), ranging from modifiable lifestyle characteristics such as diet and weight, as well as non-modifiable personal characteristics such as age, sex and family history have been identified, based on the presence of statistically significant numerical association.

Traditionally risk factors, including the CRFs, are identified by means of observational, epidemiological studies. Because such studies lack

the rigorous scientific controls characterizing experiments performed in the laboratory, often they cannot definitively distinguish between the impact of a particular risk factor and the confounding effects of other risk factors. Such considerations are especially noteworthy in epidemiological studies focusing on CHD because of the large number of suspected risk factors for this disease. In this context, it is worth noting that even the three most important risk factors for CHD, (i) cigarette smoking, (ii) hypercholesterolemia and (iii) hypertension, that have been traditionally cited for their predisposing roles leading to CHD, are unable to act as predictors of most new cases [1]. Accordingly, results of epidemiological studies identifying CHD risk factors should only be

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seriously considered when they are supported by biologically plausible mechanisms that adequately explain the relationship between the purported risk factor and the onset of CHD. Indeed, a more practical way to consider CRF is that they merely provide a conceptual framework for arriving at a coherent, practical and balanced approach for the prevention and management of CHD.

CHD is a complication of a more fundamental vascular pathology, i.e., atherosclerosis (AS), especially as it affects the coronary circulation. AS is responsible for more morbidity and mortality than any other single degenerative disease, including cancer. Its clinical expression in the form of myocardial infarction, stroke and peripheral vascular disease accounts for about 50% of all deaths in developed countries [2-4].

A key feature in the pathogenesis of this fundamental disease is the proliferation of vascular smooth muscle cells (VSMC) in the arterial intima. The end-stage atherosclerotic lesion is characterized by an abnormal focal proliferation in VSMC, which have migrated into the intima and there undergone active replication. Although this replication or proliferation is known to be influenced *in vitro* and *in vivo* by a number of blood-borne factors, designated as either "mitogens" or "growth factors" [5], in humans the stimulus for this phenomenon is unknown.

In recent years, studies have shown that various psychoactive factors may play an important, independent, role in the development of this pathology [6-9]. More recent evidence suggests that anger and "hostility" (HO) are some of the main factors, not only in coronary circulation, but also in connection with cerebrovascular disease and peripheral arterial disease, as well [10-15]. Interactions between the nervous and cardiovascular systems have been extensively studied in animal experiments as well as in human physiological laboratories. The possible role of mental arousal in provoking changes in the fundamental properties of the cardiovascular system and possibly leading to pathological events is detailed recently [16]. However, the means by which these characteristics translate into pathology at the level of the arterial wall are not understood.

In both animals and humans, electrical stimulation of the diencephalon (hypothalamus) and structures closely connected with it, such as the limbic system and tegmentum of the midbrain, may elicit "aggression" and aggressive feelings - forms of behavior which are closely linked to the underlying

trait of hostility in humans [17-21]. Electrical stimulation of the diencephalon also produces lesions in arteries (aorta) of rodents and non-human primates which bear the essential characteristics of AS plaques in humans, i.e., proliferation of VSMC, collagen and elastin synthesis and, in squirrel monkeys, lipid deposition even when diets are not high in fat content [22-24].

When young (3 months) rats on nonnal diets experiencing brief episodes of HS were investigated for a substance in the serum with growth-promoting properties for cells of the arterial wall which participate in the formation of the AS plaque, i.e., VSMC, such a substance(s) was often present. This mitogenic activity appeared to be unrelated to PDGF. More recent work has shown that when the serum of HS animals is fractionated by different concentrations of ammonium sulfate, followed by binding of the fractions to heparin-agarose columns. SDS-PAGE and silver staining of the heparinagarose bound proteins revealed that in the 30-60% ammonium sulfate fraction, there were several distinct protein bands, which were absent in the non-stimulated controls treated in the same way [25]. This finding suggested a possible link between aggressive behavior - emanating from the subcortical regions of the brain - and a cellular response in arteries contributing to atherogenesis via a serum factor which was mitogenic for these cells. Reasoning that a similar relationship might exist in human populations, the present study was undertaken among normal subjects. Our results show that upon application of a defined psychological stress, there was a significant increase of two substances in the circulating blood, which are widely believed to be atherogenic, since they induce proliferation of VSMC. These substances are: PDGF, known to be a potent growth factor for cells of mesenchymal origin, and a mitogen, entirely distinct from PDGF. The PDGF-independent mitogen was shown to induce an increase in the steady state concentration of the c-myc mRNA in the "experimentally-stressed" sera-treated VSMC.

MATERIALS AND METHODS

Materials:

[Methyl-³H]thymidine (67-77 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA). The 1.8 Kb Cla I/Eco RI human c-myc exon III probe was purchased from Oncor, and the rat 18S

rRNA probe was provided by Dr. Ira G. Wool of the University of Chicago.

Methods:

Characteristics of the subjects. The subjects consisted of 175 healthy volunteers (56% males and 44% females), aged 21-65 (mean = 41.8 ± 10.6 SD), recruited from the general public. Most (96.5%) were White and 2.7% were Black. All individuals were telephone-screened for established risk factors for atherosclerosis, current use of any medication, including aspirin, occurrence of a major life event within the past 6 months, and past history of chronic and/or debilitating disease. Ten percent of the individuals initially recruited had to be dropped for various reasons, such as not meeting the above criteria, inability to keep necessary appointments, etc. Marital status was reported as 28.3% single, 58.4% married and 13.8% divorced, separated or widowed. Exercise was reported by 50.7% as being performed more than 3 times/week and by 49.3% as less than 3 times/week. A history of smoking at least 20 cigarettes/day for 5 years or more was present in 22% of the total.

Psychological instruments. Hostility was assessed using the Cooke-Medley subscale of the MMPI [26], which is composed of fifty dichotomous (yes/no) items such as: "I have often had to take orders from someone who did not know as much as I did." The scale alpha was 0.82.

Procedures:

Prior to acceptance into the study, informed written consent to participate in each phase was obtained from all subjects. The Institutional Review Board of New York Medical College approved the study design. All participants were required to pass a complete physical examination, routine clinical laboratory procedures and an exercise stress test for cardiovascular performance. Individuals whose basal systolic blood pressure was greater than 140 mm Hg or whose diastolic blood pressure exceeded 90 mm Hg and those whose fasting serum cholesterol concentration was greater than 220 mg/dl were excluded.

Upon arrival, subjects completed the MMPI questionnaire, and were allowed to rest for a period of 15 minutes. An IV catheter was then placed in the antecubital vein of the left forearm. A BP cuff was

placed around the right arm. Catheter and cuff were arranged so as not to interfere with hand movements during the test session. Following another 15-minute period of adjustment, BP was recorded 3x from an automatic monitor at 2-minute intervals and the results (systolic/diastolic) averaged. Pulse rate was simultaneously measured. Blood samples were obtained from the antecubital vein by the same individual using indwelling catheter. Slight pressure was used for venous occlusion, with the subject seated comfortably in a relaxed position for 30 min prior to venipuncture, which was performed with a 21/gauge needle. The first few ml were discarded and the remainder set aside to clot for 30 min at room temperature (20°C). The test session described was then administered for 30 minutes. During this period all subjects were videofilmed

At the end of the test session BP and PR were recorded as before and a second blood sample obtained. Participants then completed a self-rated questionnaire dealing with the degree of frustration and anger experienced during the session. All subjects were then debriefed and notified of the "purpose" of the study. All samples were obtained at least 3 hours after a light breakfast consisting of 1 slice of dry toast and one unsweetened beverage of tea or black coffee.

Test Session. Subjects were assigned to one of two groups:

Group 1: Harassed (n=120). Individuals were asked to play a handheld electronic game (Mario Brothers) in the presence of one of the members of the research team within the time period of the session and to achieve a score, known by the team to be extremely difficult or impossible to obtain. None of the participants were familiar with the game, so did not question this objective. During the session, the interviewer periodically uttered "subtly" disparaging and critical remarks concerning the accomplishments of the participants during the session. Comments such as "I expected you to do better than that, "or" you're not even performing at the level of a ten year old child," were said in an "exasperating" manner. Most of the subjects later reported some degree of "irritation" or "anger" at these remarks.

Group 2: Frustrated (11=53) [Frustrated/Harassed are descriptive terms used to characterize individuals playing the game alone (group 2) or those in addition to playing the game, were also exposed to verbal harassment (group 1)]. The same

strategy as used for group I was employed except that the interviewer (the same individual as in group 1) encouraged the subject to improve his/her score, and scrupulously avoided criticism of any kind. All subjects later reported some degree of "frustration" with the task.

Preparation of plasma derived serum (PDS).

All blood samples were drawn from a vein in the right cubital fossa, before and after administration of psychological test. Each 10-ml of blood was immediately placed into a chilled 50 ml Falcon centrifuge tube containing 1 ml mixture of sodium citrate, EDTA, theophylline and PGE₁ (antiplatelet antiaggregating agents). Plasma was first separated by centrifugation at 3500 rpm 4°C for 15 min. and spun another 30 min at 4°C 16,000 rpm in rotor SS34, RC Sorvall centrifuge in order to remove platelets. Supernatant was transferred to 50 ml Kimax glass tubes and 4% of 1 M CaCl₂ was added with incubation at 37°C for 2 hours in a water bath. After clot developed, the solution was spun for 30 min at 4°C/16000 rpm. The supernatant was transferred to prepared dialysis tubing and dialyzed in the cold room at 4°C against freshly prepared Dulbecco's PBS containing CaCl₂, with one buffer change after 2 hours. After dialysis, the supernatant was heat-inactivated at 56°C for 30 min. The supernatant was centrifuged and filtered through sterile 0.45 µm Millex-HA units, aliquoted, and stored at -70°C. PDS samples prepared in this way have been shown to be free of PDGF by radioimmunoassay.

Determination of serum PDGF. Serum concentrations of PDGF were measured with a sensitive radioimmunoassay procedure (Amersham International Pic, Amersham, UK). The assay was based on competitive binding between serum PDGF and radioactively labeled recombinant PDGF, for a PDGF-specific antibody. As reported by the manufacturer, cross-reactivity with other growth factors (EGF, TGF-13, bFGF) was less than 0.1%. Reproducibility was given as coefficient of variation = 6.5% for intra-assay and 10.3-23.5% for inter-assay determination, depending on the concentration assayed. Consistency in accuracy of PDGF determination with different lots of the commercial kit used was evaluated on three separate occasions by measuring samples of known PDGF concentration and agreed with each other within a

range of 7-10%.

Stability of serum PDGF was ascertained on two occasions by adding a pre-determined amount of PDGF to sera, freezing and storing the "supplemented" sera at -85°C, followed by thawing the stored samples at monthly intervals (up to 4 months), assaying PDGF by RIA and showing that PDGF recovery was within the error limits established for the assay. Using this procedure, a total of 6 samples were tested at random. The presence of inhibitors of PDGF or substances that interfere with the PDGF assay was essentially ruled out.

Serum Cortisol Concentration. Cortisol was assayed by a solid-phase radioimmunoassay, based on competition of cortisol in subject serum with ¹²⁵I-labeled cortisol for antibody sites over a fixed time (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA 90045). This assay was used as a stress marker.

Arterial Blood Pressure (BP) and Pulse Rate (PR). The measurements were taken in the right arm with an Automated Blood Pressure and Pulse Rate SD-700A Series Monitor System (Industrial and Biomedical Sensor Corp, Waltham, MA) based on the low frequency vibration detection principle for BP, and conversion to oscillometric pressure surges to average values, for PR. Three separate measurements for systolic and diastolic BP and PR were obtained at 2 min intervals with the subjects in a relaxed sitting position and averages computed for the data.

Total cholesterol and HDL-cholesterol assays. Values were obtained using the Abbot Vision System (Abbot Laboratories, Abbot Park, IL 60064) with total cholesterol assayed by the coupled cholesterol oxidase-peroxidase method [27], and HDL cholesterol, via initial precipitation of LDL and VLDL by magnesium and dextran sulfate, based on the method of Artiss and Zak [28].

Cell culture. Primary cultures of VSMC were obtained by outgrowth of explants from human thoracic aorta immediately obtained from young accident victims (20-30 years) within 6 hours of death, in cooperation with the organ donor service of this institution, as follows: adventitial layers were first removed with sharp forceps, and the exposed endothelium was scraped using a blade.

The strips of the middle third of the tunica media was dissected and incubated for 35 min in Minimal Essential Medium supplemented with 0.2 mM Ca, plus elastase (type III, 15 units/ml), collagenase (type I, 200 units/ml), soy bean trypsin inhibitor (0.4 mg/ml), bovine serum albumin (2 mg/ml). After incubation, tunica media was washed twice with Earle's balanced salt solution, minced, and resuspended in culture medium containing RPMI 1640, 1% penicillin, 1% streptomycin, 1% glutamine, 0.1% insulin/ trans - ferrin/ selenium (ITS), and 20% heat-inactivated fetal bovine serum. Primary VSMC were grown at 37°C with 5% CO₂ in air and reached confluence after 27 days. The identity of VSMC was confirmed by immunofluorescent staining. Cells in primary culture were treated with 0.05% trypsin-EDTA and dispersed into T-75 cm² flasks at a density of 1.0x10⁶/75 cm². The VSMC were subcultured every 7 days. Cells between passage 4-9 were used in all experiments. Viability was estimated to be 100% by the trypan blue exclusion.

Cell growth. The effects of human PDS on VSMC growth were monitored by the increase in cell number. Cells were seeded at a density of 0.5 x 10⁴/cm² and maintained for 3 days in culture medium containing 20% heat-inactivated FBS. They were washed three times with serum-free medium (RPMI 1640) and kept in the same medium supplemented with 0.5% albumin for 2 days in order to induce cellular quiescence, after which they were switched to media supplemented with 5% FBS or 5% PDS and maintained in these different experimental solutions for an additional 5 days. Cell numbers were determined by trypsinization of adherent cells and counting with a hemacytometer on days 2 and 5. Triplicate wells were counted for each time point of all experiments.

[³ H]Thymidine Incorporation. Cells were seeded at a density of 2.25 x 10⁴/cm² and cultured for 3 days in 0.25 ml RPMI 1640 medium supplemented with 20% heat-inactivated FBS. Cellular quiescence was achieved by switching and maintaining them for 2 days in serum-free medium plus 0.5% albumin. Cells were then exposed to the RPMI 1640 supplemented with 5% FBS or 5% PDS and incubated for 24 h. Prior to cell harvest, cells were incubated for an additional 2 h in the same, freshly prepared FBS or PDS

media, supplemented with 2 p.Ci [methyl-³H]thymidine. The experiments were terminated by washing cells with Dulbecco's phosphate buffered saline, followed by 10% trichloroacetic acid, and extraction of the DNA with 0.1% SDS/0.5N NaOH. The radioactivity of an aliquot of the extract was used to measure [³H]thymidine incorporation by scintillation counting. Analysis was performed in triplicate.

RNA Isolation and Northern Blot Analysis. These studies were done using a subpopulation of subjects whose pre- and post-stress sera gave the most pronounced differential increase in thymidine incorporation when added to cultured VSMC. Such increases presumably were due to changes in "experimentally-induced" PDC F-independent mitogenic activity. A total of six sets of sera, with 5 from group 1 and 1 from group 2, were used for these experiments. Cells were seeded in 75 cm² flasks containing 20% FBS until 80%-85% confluence was reached. They were switched to a serum free media for 2 days, after which they were cultured in 5% FBS or 5% PDS for 15 min, 30 min, 1, 2 and 24 h, respectively, and harvested. Total RNA was prepared from VSMC using commercial reagents (TRSOLVTM, BIOTECX LABORATORIES, INC). RNA (15 lig) were denatured with glyoxal and dimethyl sulfoxide, electrophoresed in 1% agarose, and transferred to nitrocellulose membrane (OptiBind, 0.45 urn, Schleicher and Schuell) using a LKB Vacugene system, and baked for 2 h at 80°C. Prehybridization was performed overnight at 42°C in 50% formamide, 5X SSC, 5X Denhardt's solution, 50 mM phosphate buffer, pH 6.5 and 250 µg/ml denatured salmon sperm DNA. Following prehybridization, hybridizations were carried out at 42°C for 16-20 h in 50% formamide, 5X SSC, 1x Denhardt's solution, 20 mM phosphate buffer, pH 6.5, 10% dextran sulfate and either the ³²P-labeled cmyc or 18S rRNA probes, prepared by using the random primer labeling method. The membranes were washed twice with 2X SSC, 0.1% SDS at room temperature for 10 min, followed by a 45 min wash at 55°C in the above solution, and another wash for 45 min at 55°C in 0.1x SSC/0.1% SDS. The filters were dried and exposed to Kodak XAR-5 film sandwiched between two intensifying screens. Scanning was performed on the autoradiogram using a Hoefer Scientific Instrument GS-300 scanning densitometer (San Francisco, CA).

Statistics. Statistical calculations were done using the Student's t-test to analyze differences in the measured variable. Among groups, comparisons were made by contrasting paired means of the variable in question, between before and after psychological stress.

RESULTS

Properties of Cultured VSMC. Primary VSMC grown at 37°C with 5% CO₂ reached confluence after 27 days. They showed characteristic multi-layered, spindle-shaped "hills and valleys" pattern, and were confirmed to be smooth muscle cells by immunofluorescent staining using anti- α -smooth muscle actin. When the primary antibody was replaced with normal mouse ascites fluid, staining of the cells could not be demonstrated (data not shown). When cultured in media supplemented with 20% heat-inactivated FBS, VSMC grew exponentially from the third to the tenth day, with an approximate doubling time of 31.0 ± 0.6 h (based on triplicate determinations from passage 7 of three individual human specimens, Figure 1). These data agreed closely with the peak of DNA synthesis (estimated to be 26 h, data not shown). In general, early passage cells [5-7] show a two to three times greater proliferative potential than late passage cells ([8-9], Figure 1). Correspondingly the doubling time of late passage cells increased by 20% to 37 h.

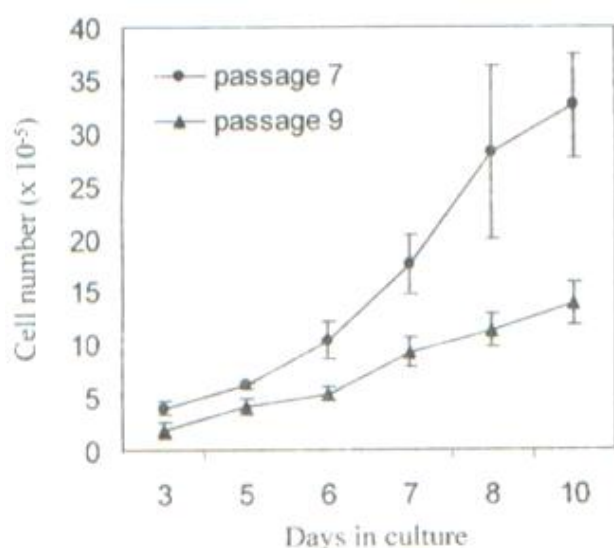


Fig. 1. Proliferation of VSMC in FBS-supplemented media. Cells from three individual explants were cultured with 20% heat-inactivated FBS. Aliquots were counted at the times indicated. Results were the mean and standard error of three experiments, each determined in triplicate.

Compared to FBS, PDS was only 10% as active in supporting the growth of VSMC. However, compared to cultures maintained in media supplemented with 0.5% BSA only, growth of the PDS-fortified cultures was stimulated by 3-6-fold, reaching peak of growth after 25-30 h, based on either an increase in cell number or the incorporation of [³H] thymidine into DNA.

Changes in VSMC Proliferation, PDGF, cortisol, blood pressure and heart rate in "Harassed" and "Frustrated" Subjects. To investigate whether a relationship exists between "stress" and atherogenesis and whether the effects of "stress" are mediated through blood-borne substances, we assayed for a number of parameters in sera after the subjects were exposed to one of 2 "stress" challenges. The challenges consisted of a single anger-provoking stress: a cognitive task with superimposed verbal harassment (group 1), and the cognitive task without harassment (group 2). Mitogenic activity was determined via the effect of adding PDS (PDGF-depleted) from treated subjects to growing cultures of VSMC and measuring incorporation of radioactive thymidine or increase in cell number, as described in Materials and Methods. Blood pressure (BP), heart rate (FfR), cortisol, and serum total and HDL cholesterol were also evaluated. All measurements were made immediately before (baseline) and after a 30 min interval.

Analysis of the data revealed that there were 33% of subjects in group 1 with an increase of thymidine incorporation 15% or greater than baseline, versus 21% in group 2 (Figure 2). The average increases were 45% and 30%. Increased proliferation of these VSMC in response to added sera from "stressed" subjects in an essentially nonsmoking population, suggests that repeated episodic provocations eliciting a similar serum response could make an important contribution to the development of atherosclerosis in susceptible individuals.

The values of PDGF concentration in the prestress sera of 173 healthy subjects range from a low of 1.4 ng/ml to a high of 8.4 ng/ml. The distribution is skewed and clearly non-Gaussian (data not shown). A lower reference limit was not computed because approximately 46% of the values were in the range 1.4-2.4 ng/ml. However, although low values of serum PDGF concentration may ultimately turn out to be of importance, most of the current interest in this polypeptide as an important growth factor is in its higher values. The upper reference limit was found to be 6.5 ng/ml, with a 90% confidence

interval of 5.9-8.4 ng/ml. In comparing the PDGF values of the pre- and post-stress sera, approximately 50% of the subjects showed an increase in PDGF of $>15\%$ (Figure 2). In a few studies, elevated levels of circulating PDGF have been implicated in association with atherosclerotic disease, both in plasma and in serum [29-31].

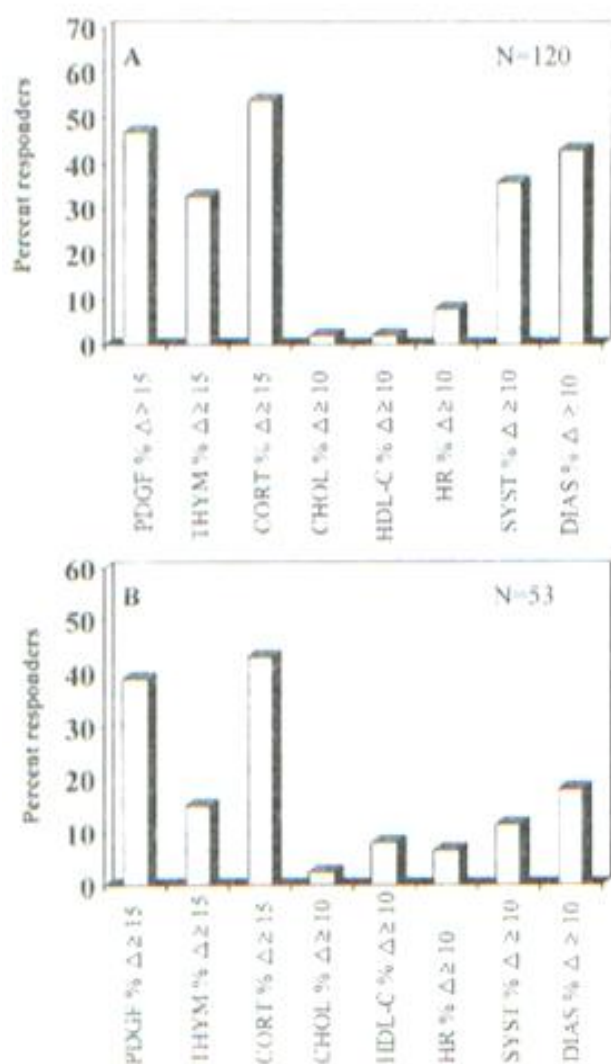


Fig. 2. Biochemical changes in blood sera following cognitive challenge. The percentage of individuals responding to two separate "stress" applications on N=120 and 53 subjects are shown. "Responders" were defined according to the value above pre-stress levels on the abscissa for each parameter studied. PDGF and cortisol values were measured in serum by conventional RIA techniques. Panel A. Group 1: "harassed" subjects (N=120). Panel B. Group 2: "frustrated" subjects (N=53). Thym = incorporation of [3 H-methyl] thymidine by human VSMC in cell culture, as a proliferation assay related to the PDGF-independent mitogen in the study. Serum total and HDL-cholesterol were measured by the Abbot Vision System. HR and BP were determined by an automated blood pressure and pulse rate monitor system.

Total pre-stress cholesterol values (n=173) ranged from 93 to 320 mg/dl with a mean of $192 \text{ mg/dL} \pm 38 \text{ mg/dl}$ SD, whereas concentration of HDL cholesterol varied from a minimum of 24 to a maximum of 78 mg/dl (n=173), with a mean of $45 \pm 12 \text{ mg/dl}$ SD. Total and HDL cholesterol showed slight changes only as a result of stress in both groups.

Data for the pre-stress cardiovascular parameters (n=175) were as follows: Systolic BP (range, 85 to 159 mm Hg with mean of $112 \pm 13 \text{ mm Hg}$ SD); diastolic BP (range of 49 to 115 mm Hg with a mean of $77 \pm 11 \text{ mm Hg}$ SD) and PR (range 42 to 101 beats per minute (bpm) with a mean of $69 \pm 10 \text{ bpm}$ SD). BP and cortisol were elevated by approximately 10% in group 2 while HR showed slight changes only as a result of stress in both groups.

Sera of "Psychologically-Stressed" Subjects on Growth, DNA synthesis, and c-Myc Protooncogene Expression in Cultured VSMC. To determine whether PDS prepared from pre- and post-stress subjects had differential effects on cultured VSMC, cells were incubated with various concentrations of pre- and post-stress PDS prepared from the same individual, for up to 5 days. Results of cultures maintained with 5% PDS are presented in Table 1. Clearly the post-stress PDS was more effective in inducing VSMC proliferation, as measured by increases in cell count. The difference was statistically significant for the day 5 cultures, $P < 0.05$ (Table 1, n=6). Similar results were obtained when proliferation was measured by incorporation of radioactive thymidine into DNA, $P < 0.05$ (Table 1, n = 6). The cell count increase correlated well with changes in thymidine uptake (correlation coefficient, $r = 0.745$, $P < 0.0005$).

Since previous studies have shown a correlation between cellular proliferation, DNA synthesis, and the expression of c-myc in a number of mammalian cells [32-34], we determined c-myc expression in control and treated-cells using Northern Blot analysis. Total RNA was isolated from VSMC and probed with a labeled c-myc exon-III. The 18S rRNA was used as an internal control to verify the precision of RNA loading. VSMC cultured in serum free medium and switched to 5% FBS had peak c-myc expression at 0.5 h (data not shown), with no corresponding change in the expression of the 18S rRNA. When c-myc changes in cells cultured with pre- and post-stress PDS were compared, a significant difference, as evidenced by the greater post-PDS-elicited c-myc mRNA

increase, was observed. In the majority of the cases tested (5/6), the c-myc mRNA expression was consistently less when cells were maintained in the pre-stress sera (Table 1, n=5, P<0.05). Such a result coincided with the observed cellular proliferation and radioactive thymidine incorporation data. In one individual whose sera elicited a large differential thymidine incorporation response in VSMC, no corresponding increase in c-myc was observed. Instead, the pre-stress PDS gave higher c-myc mRNA value, compared with the post-stress PDS.

DISCUSSION

The data show that, among those individuals with altered emotional states, there emerged a statistically significant relationship between a subpopulations in this group and increases in the serum concentration of two mitogenic substances: PDGF and a non-PDGF mitogen for cultured human VSMC in their blood samples, i.e. approximately 3040% of them responded with substantial increases of the blood PDGF concentrations, as well as a PDGF independent mitogen, when compared with their own pre-stress levels. In the same study there was little changes in the traditional risk factors, such as hypercholesteremia or hypertension. Serum cortisol concentrations, however, did increase in many of these subjects, indicating that they underwent a stress response. Results of these studies, although preliminary, tend to support findings from epidemiologic studies, indicating that individuals characterized by a more active or aggressive personality are more prone to the development of coronary heart disease, the major complication of AS, than their relaxed controls [6-21,35].

The nature of the substance or substances in the serum responsible for the increase of mitogenic activity of cultured VSMC cannot be determined from the data of this study. Serum contains a number of growth factors for VSMC, of which PDGF is the most potent [5, 36]. In this investigation, PDGF could be ruled out. Substances other than growth factors in the serum also possess mitogenic properties for these cells [20-24, 37]. The exact identity of the molecular nature of this substance awaits further work.

The precise mechanism by which altered emotional states could result in an increase in serum mitogenic activity for cells that participate in the atherogenic process remain to be determined. Work in

experimental animals (rats and the nonhuman primates, *S. sciureus*) suggests that the diencephalon may be involved. In these experiments, electrical stimulation of the hypothalamus gives rise to a series of complex steps which culminate in the proliferation of VSMC, both *in vitro* and *in vivo* and in the arterial lesions of atherosclerosis, as well as in agitated behavior when the animals are not under anesthesia. These results have been reviewed in earlier publications [20-24]. Assuming a similarity in the human situation, the present investigation focuses on the increase of serum mitogenic activity alone - although the means by which this response is achieved is currently unknown.

Implications for Atherogenesis and CHD: Complexities of Atherosclerosis and CHD.

Although CHD accounts for about 27% of the 21 million deaths each year among Americans, with AS being the principal cause [38], there are controversial methodological issues regarding the diagnosis of AS [39], since at times those free of clinical disease have been found with more severe coronary atherosclerosis than others with clinically manifest CHD [40]. Traditionally there are three dominant features characterizing atherosclerotic lesions: (i) proliferation of smooth muscle cells which accumulate together with a variable but large number of blood derived macrophages; (ii) deposition of large amounts of connective tissue matrix proteins such as collagen, elastic fibers and proteoglycan around the smooth muscle cells; and (iii) lipid accumulation in the form of foam cells within smooth muscle cells and macrophages, and as deposits within the extracellular matrix surrounding these cells. Some form of endothelial injury is believed to contribute to the initiation of atherosclerosis.

Many aspects concerning AS and CHD remain unanswered. For example, the process of smooth muscle proliferation and lipid accumulation might be expected to be linear with time. But angiographic studies show that the progression of coronary artery disease in humans is neither linear nor predictable [41]. New high grade lesions often appear in segments of artery that were normal at previous angiographic examinations [42]; and it is impossible to foresee the site of subsequent occlusion causing infarction. One possible explanation for this unpredictable and episodic progress is the occurrence of thrombosis, as a complication of the basic atherosclerotic process, leading to intermittent

Table 1. Effects of pre- and post-challenge PDS on proliferation, thymidine incorporation and c-myc mRNA levels of VSMC.

Experiment One: Changes in Cell Number, n=6	Day 2 in Culture Cell Number x 10 ⁻⁵	Day 5 in culture Cell Number x 10 ⁻⁵
Pre-stress PDS	0.60 ± 0.3	0.75 ± 0.3 ^a
Post-stress PDS	0.73 ± 0.3	1.30 ± 0.4
Experiment Two: Changes in DNA synthesis, n=6		Day 5 culture ³ H-thymidine incorporation (cpm)
Pre-stress PDS		1,250 ± 150 ^b
Post-stress PDS		1,950 ± 210
Experiment Three: Changes in c-myc mRNA, n=5	c-myc mRNA/18S rRNA (arbitrary units) 0.5 h in culture	c-myc mRNA/18S rRNA (arbitrary units) 1.0 h in culture
Pre-stress PDS	1.00 ± 0.2 ^c	2.5 ± 0.6 ^c
Post-stress PDS	1.95 ± 0.5	3.1 ± 0.8

^a Effects of pre- and post-challenge PDS on proliferation of VSMC. The difference was statistically significant for the day 5 cultures, P<0.05. ^bEffects of pre- and post-challenge PDS on [³H]thymidine incorporation into DNA in day 5 cultures of VSMC, P<0.05. The cell count increase in Experiment 1 correlated well with changes in thymidine uptake (correlation coefficient, r=0.745, P<0.0005). ^cNorthern blot analysis of c-myc mRNA level changes in VSMC cultured in the presence of pre and post-challenge 5% PDS, P<0.05. The 18S rRNA was used as an internal control. The results of densitometric scans are shown

plaque growth [43]. Moreover, as pointed out earlier, the three most important risk factors for CHD-(i) cigarette smoking, (ii) hypercholesterolemia, and (iii) hypertension are unable to account for its mechanism, or act as predictors of most new cases [1]. For example, hypertension has been shown to be predictive of CHD in the elderly [44], whereas the relative risk attached to cigarette smoking appear to diminish with advancing age in some and not in all studies [45]. The prediction of CHD by serum cholesterol appears to decline with advancing age [46].

Psychosocial/behavioral factors as modulators of risk factors for CHD. What could account for these complex CHD prediction outcomes based on traditional risk factor analysis? How is it that the impact and magnitude of a risk factor characterized in a given locale may differ significantly when examined in a different region, country, and culture? The explanation for such divergence in human response to a given risk factor, apart from the often-considered confounders such as ethnicity, gender, etc., may lie in the influence of psychosocial and behavioral traits.

There is accumulating evidence that psychosocial

factors can play a role in modulating response to a universally accepted risk, and may have an especially critical contribution in situations involving risks of marginal significance. In relation to the development of AS, abnormal platelet function tending to release platelet factors such as PDGF, in the circulating blood, which have become very important in AS, especially in CHD patients and subjects with cerebrovascular disease, has been observed numerous times during psychological stress. This is true of both animals and humans, thus establishing a possible mechanistic link between these events and impinging upon AS. Platelet activation, which is considered to be important in atherogenesis and cardiac fatalities, has been reported to be induced *in vivo* to different stressful events such as surgery and dynamic exercise. Mental stress (such as emotional provocation or that induced by taking the Stroop's Colour Word Conflict Test) has been shown to increase platelet activation as indicated by aggregability tests, probably because of the post-stress release of vasoactive compounds from platelets [47, 48]. Similarly, feelings of anger and anxiety determine blood pressure and hypertension and hence may increase the risk for essential hypertension [49-

52]. In fact perceptions of risk and adverse health is significantly associated with the actual risk for heart disease [53-56]. Grossarth-Maticek and coworkers provided evidence for the powerful effect personality and stress exerted on a person's likelihood of dying of cancer and coronary heart disease, and for their ability to interact strongly with more widely studied risk factors, such as smoking [57].

The apparent link existing between psychosocial/behavioral factors and cellular proliferation, as exemplified by SMC or even cancer cell proliferation, may be biologically plausible. One possibility is that heightened neural activity in an untimely manner may induce proliferation of cancerous or VSMC cells via a stress-induced neuroendocrine mechanism, or through changes in hormonal and immune status and hence functions [58, 59]. There is evidence that anger-suppressing personality type is associated with a specific antibody profile and that lymphocyte level is depressed in subjects who have recently suffered grave emotional losses, e.g., death of a spouse. Animal studies have demonstrated that the sympathetic arousal invoked by psychosocial variables, commonly known as "stress", is accompanied by endothelial dysfunction of the coronary arteries and aorta, as judged by enhanced immunoglobulin G incorporation and endothelial cell replication [60]. The demonstration by Pettersson et al. [61] that chloralose-induced endothelial cell injury in rabbits is effectively blocked by metoprolol, a β_1 -adrenergic antagonist, further supports the notion that increased sympathetic nervous activity and catecholamines are intimately involved in aortic endothelial cell damage and possibly atherogenesis.

Stress-induced blood-borne atherogenic factors in relation to other purported risk factors for CHD.

The results of this study may have significant implications on the multifactorial nature of CHD, particularly in relation to such esoteric risk factors as noise, behavior, and the widely publicized and as yet unconfirmed exposure to environmental tobacco smoke (ETS). A single anger-provoking stress was sufficient to induce increases in the blood of two substances that are proliferative for VSMC, in approximately 30-45% of normal human subjects, aged 21-65. Moreover, based on results of the thymidine incorporation assay, there seemed to be more of the PDGF-independent mitogen in blood of "harassed" individuals, suggesting that the elicitation

of these substances may be dependent on the degree of anger that an individual's experiences. As common observation shows, the exposure to ETS, because of distinct odor of ETS and the likelihood that it could act as an irritant for some individuals, may elicit a strong anger-provoking response in some individuals, e.g., in public places such as restaurants and bus depots, in the workplace, or even the home. Our findings thus raise the possibility that a similar phenomenon occurs in individuals exposed to ETS, namely that they may respond to the presence of ETS, with elevated blood concentration of PDGF and an independent serum mitogen for VSMC, not necessarily because of the exposure to the physical presence of the smoke, but to the anger-provoking stress which may accompany this exposure. Additional experiments will be required to clarify the respective roles of ETS and the stress with which it may be associated in the induction of atherogenic growth factors in the blood of ETS-exposed individuals.

CONCLUSION

In conclusion, data from this study, performed with 173 healthy human subjects, lends support to the role of psychological factors in atherogenesis, by revealing that increases of a serum mitogen for cultured human VSMC - a key component of the pathogenesis of atherosclerosis - may occur under certain conditions in subjects with altered emotional states.

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