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Pain-Related Activation of Leukocyte Cellular Adhesion Molecules: Preliminary Findings

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Key Words
Cellular adhesion molecules • Cortisol • Epinephrine • Inflammation • Integrins • Pain • Stress

Abstract
Background/Aims: Acute adrenergic stressors have been found to activate neuroendocrine pathways that can alter leukocyte migration and activity. Leukocyte migration is known to affect the pathophysiology of inflammatory disease processes. This study examined the effects of acute experimental pain on catecholamine and cortisol levels and leukocyte expression of cellular adhesion molecules. Methods: Healthy subjects (n = 10) underwent 45 min of acute experimental pain using earlobe electrical stimulation. Measures included sensory and affective pain responses, perceived stress, circulating levels of catecholamines, cortisol, and expression of integrin (CD11a+) cellular adhesion molecules on leukocyte subsets. Data were collected at baseline, after 22.5 and 45 min of pain, and 180 min after pain cessation. Results: Experimental pain acutely increased circulating levels of epinephrine, along with increases in the number of CD8+CD11a+ leukocytes and the density of CD11a molecules on CD8+ cells. Positive correlations were found between pain and stress scores, and the number of CD8+CD11a+ leukocytes. Conclusion: Acute pain induces elevated cellular adhesion molecule expression on leukocytes, which has possible implications for increasing leukocyte infiltration and disease exacerbation in patient populations with inflammatory syndromes.

Introduction
The pathophysiological changes associated with nociception have the potential to affect leukocyte infiltration with implications for increased pathology in diseases such as rheumatoid arthritis, inflammatory bowel diseases, and vascular atherosclerosis [1]. Pain is known to evoke a neurohormonal stress response as a consequence of afferent neural signals conducted to central sites of sensory, autonomic, and neuroendocrine integration [2]. There is concurrent activation of efferent adrenergic fibers innervating critical bodily organs including lymphoid tissue [3] and the hypothalamic-pituitary-adrenal and sympathetic-adrenomedullary stress axes, resulting in the production of catecholamine [epinephrine (EPI) and norepinephrine (NE) and cortisol] molecules. These major outflow pathways of the central nervous system comprise crucial neural and humoral mechanisms linking pain to immune alterations [4].
Patients and Methods

Sample
A sample of 10 (5 male and 5 female) volunteers was recruited and study inclusion criteria included good health and age of 18–31 years. A power analysis was conducted on data from a study examining leukocyte integrin CAM responses (CD11a) to an adrenergic stressor [8]. Based on the data (in control subjects) for CAM expression (mean number of CD11a molecules bound per cell) in response to exercise stress of Mills et al. [8], a sample size of 10 will have 80% power to detect a difference in means (before vs. after treatment) of 3,277, assuming a standard deviation of differences of 2,725 using a paired t test with a 0.05 two-sided significance level. Exclusion criteria were: pregnancy; ingestion of medications influencing catecholamines, pain perception, or immune status; presence of pain syndromes, immune compromise, or acute infection within 4 weeks prior to study session. The study was approved by the Medical Institutional Review Board (University of California at Los Angeles), and each subject gave informed consent before the start of the study. Subjects were financially compensated for their participation.

Procedures
Pain Induction
All study sessions were performed at the General Clinical Research Center, University of California, Los Angeles, and began at 08.00 with the application of automated blood pressure cuff, electrocardiogram, pulse oximeter and insertion of a 22-gauge angiocatheter into the subject’s randomly chosen antecubital fossa for blood sampling.

Pain was induced after a 45-min rest period by applying voltage with an earlobe algesimeter (SD9 Square Pulse Generator, Grass Products). This pain model was chosen because electrical stimulation is known to provoke pain-related immune responses [6], and its application to the small skin area of the earlobe allowed careful assessment to detect tissue damage. Stimulation time course and intensity were determined to allow for the development of autonomic responses, gene transcription, and to avoid undue subject risk and burden.

Electroconductive gel was applied over the randomly chosen earlobe and the ear clip electrodes were applied. Electrical pulses (frequency 0.7 pulses per second) of 14-ms duration were increased gradually (every 1–2 s) by 2-volt increments (starting at 0 V) as previously described [9]. Subjects were instructed to attain a numeric rating scale pain score of 0 (0 = no pain, 10 = worst possible pain), and to maintain it continuously for 45 min. To increase the perceived stress associated with the pain, at random intervals throughout the stimulation period, investigators increased voltage acutely and briefly to maximum subject tolerance. Subjects were instructed to immediately notify the researchers when increased ear pain was detected, at which time the subject was informed that a ‘tolerance test’ was underway, and that voltage would be increased every 1–2 s until the subject stated it was no longer tolerable, then immediately reduced back to the level corresponding to a pain score of 6. Vital signs were recorded every 5 min; pain response and stress levels were assessed concurrently with blood sampling at baseline (T1), pain + 22.5 min (T2), pain + 45 min (T3), and 180 min after pain (T4), after which subjects were discharged.

Pain and Stress Measures
The average voltage and milliamperage (Digital Multimeter, model Fluke 733, Grainger, Los Angeles, Calif., USA) to which subjects were exposed was measured between T1 and T3 (45 min, sampled once per minute).

Pain intensity was regulated at a uniform level for all subjects using an 11-point numeric rating scale, a well-demonstrated approach [10]. In addition, the short-form McGill Pain Questionnaire (SF-MPQ), an assessment instrument with established reliability and validity that is used widely in clinical and experimental settings, was utilized to assess sensory and affective pain responses [10].

Perceived stress during painful stimulation was assessed using a 100-mm visual analogue scale (VAS) with the following anchors at each end: ‘I feel no stress’——’I feel extremely stressed’. VASs have well-established reliability and validity in the assessment of symptoms such as pain and stress [10].

Hormone and Cardiovascular Measures
EPI and NE were measured at T1 and T3 with high performance liquid chromatography (Bio-Rad Chromatopac model CR501, Specialty Laboratories, Valencia, Calif., USA). The analytical sensitivity for EPI was 5 pg/ml with a dynamic range of 5–4,000 pg/ml; the analytical sensitivity for NE was 17 pg/ml with a dynamic range of 15–1,000 pg/ml. Plasma cortisol was measured at all four data collection points (T1–T4) using a commercially available ELISA kit (Nichols Institute Diagnostics, San Clemente, Calif., USA); interassay coefficient of variation ≤17.4% and intra-assay coefficient of variation ≥8.7%). Pulse rate and mean arterial pressure were measured as additional indices of sympathetic stimulation.
The number of days from the last day of the menstrual period to the study date was recorded for each female subject. History of oral contraceptive use was noted.

CAM Measures
The number of CD11a+ cells and the density of CD11a expression were determined using the FACSCalibur flow cytometer and CellQuest software on blood kept at ambient temperature. Blood specimens were stained with monoclonal antibodies conjugated with either fluorescein isothiocyanate, phycoerythrin (PE), peridinin chlorophyll protein, or allophycocyanin and lysed by FACS Lysing Solution (BD Biosciences PharMingen, San Jose, Calif., USA). Instrument fluorescence compensation was performed using CaliBRITE beads and FACSComp software. A complete blood count was performed using an Act Diff CBC analyzer (Beckman Coulter). The results were presented as percentages of CD4-, CD8-, CD14-, and CD11a-expressing cells, and the numbers (cell/mm$^3$) of CD4-, CD8-, and CD14-positive cells that also expressed CD11a were calculated from the total number of lymphocytes and monocytes.

For density quantitation of CD11a on CD4+ and CD8+ lymphocytes, and on CD14+ monocytes, a flow-cytometric estimation of antibodies bound/cell was performed using Quantibrite PE beads (BD Biosciences). The Quantibrite PE beads were run at the same instrument settings as the assay and the FL2 axis was converted into the number of PE molecules bound/cell.

Statistical Analysis
Descriptive statistics were calculated for each variable at each time point. Pearson’s r, or for non-normally distributed data, Spearman’s p was calculated to analyze relationships among variables. Analyzed responses included the changes from baseline (T1) to each other time point (T2, T3, and T4) for the SF-MPQ scores, stress VAS scores, pulse rate, mean arterial pressure, hormones, and CAMs utilizing repeated measures analysis of variance. Post-hoc testing of specific changes (i.e. T1 vs. T2, T3, T4, across all pairs) employed Student’s paired t test, and in the case of non-normally distributed data, Wilcoxon’s signed-rank test (SPSS statistical software, version 11.0.1). Statistical significance was set at an α level of 0.05.

Results
Sample Demographic Data
Mean subject data included the following: age 25 years (SD = 3.6); height 168.5 cm (8.2); weight 67.7 kg (7.7), and body mass index 23.9 (1.6). There were 3 Caucasian, 2 African-American, and 4 Latino subjects. No relationship was found between the number of days from the last day of the menstrual period to the study date and any other study variable. One female subject took oral contraceptive pills, but no effects were observed on study results.

Pain and Stress
All subjects demonstrated skill in quantifying the pain intensity, and achieved and maintained a numeric rating scale score of ‘6’ for the 45-min stimulation period, and underwent random tolerance testing without difficulty. Pain and stress measures were significantly elevated from baseline during stimulation (table 1). Mean SF-MPQ total scores, SF-MPQ sensory scores, and stress VAS scores were higher at T3 than T2; all variables returned to baseline values at T4. The mean SF-MPQ total scores were positively correlated with mean stress VAS scores at T2 [r(8) = 0.60, p = 0.036] and T3 [r(8) = 0.66, p = 0.049].

Table 1. Pain, stress and immune variables

| Parameters                          | Time points | T1       | T2            | T3            | T4            |
|------------------------------------|-------------|----------|---------------|---------------|---------------|
| SF-MPQ, points                     |             |          |               |               |               |
| Total                              |             | 0        | 14.3 (4.5)$^a$ | 18.3 (4.4)$^b$ |               |
| Sensory                            |             | 0        | 12.9 (4.5)$^a$ | 16.5 (4.5)$^c$ | 0             |
| Affective                          |             | 0        | 1.4 (1.4)$^d$  | 1.8 (2.1)     | 0             |
| Stress VAS, mm                     |             | 0        | 33 (17.3)$^a$  | 44 (19.5)$^e$  | 1.4 (2.9)     |
| MAP, mm Hg                         | 77 (8.6)    | 78 (10.9) | 80 (8.2)      | 76 (5.1)      |               |
| PR, b.p.m.                         | 61 (7.6)    | 58 (6.3)  | 62 (7.0)      | 68 (8.2)      |               |
| EPI, pg/ml                         | 7.8 (2.5)   | –        | 14.6 (7.8)$^f$ | –             |               |
| NE, pg/ml                          | 125.7 (49.6)| –        | 154.4 (80.0)   | –             |               |
| Cortisol, µ/ml                     | 9.9 (4.9)   | 9.6 (4.4) | 9.0 (3.9)     | 8.7 (2.4)     |               |
| CD8+CD11a+, cells/µl              | 553.2 (116.9)| 630.4 (157.6)$^f$ | 602.4 (110.2)$^g$ | 560.8 (132.3) |               |

Values are means (SD). MAP = Mean arterial blood pressure; PR = pulse rate.

$p = 0.005$ vs. T1 (Z = –2.8); $^b$ p = 0.008 vs. T2 (Z = –2.7); $^c$ p = 0.016 vs. T2 (Z = –2.7); $^d$ p = 0.016 vs. T1 (Z = –2.7); $^e$ p = 0.007 vs. T1 (Z = –2.7); $^f$ p = 0.028 vs. T1 (Z = –2.2); $^g$ p = 0.017 vs. T1 (Z = –2.4). For further information, see the text.
Hormone and Cardiovascular Measures

The mean plasma level of EPI increased significantly in response to the nociceptive stressor at T3 (table 1). Neither NE, cortisol, pulse rate, nor mean arterial blood pressure changed during the pain stressor.

CAM Expression

The mean number of CD8+CD11a+ cells/µl increased during pain compared to baseline [F(3,27) = 3.71, p = 0.023; table 1]; no change was seen for CD4+ or CD14+ cells. Likewise, the density of CD11a molecules on CD8+CD11a+ cells increased significantly from baseline during pain [F(3,27) = 4.19, p = 0.015; fig. 1]. The number of CD8+CD11a+ cells at T2 was significantly correlated with the stress VAS scores at T2 [r(8) = 0.82, p = 0.004], and T3 [r(8) = 0.80, p = 0.005]. Neither the catecholamine, cortisol, nor cardiovascular measures were correlated with the mean number of CD8+CD11a+ cells, or the mean density of CD11a expression on those cells at any data collection time point.

Discussion

Thus healthy volunteers were subjected to acute experimental pain, and the number of circulating catecholamine molecules and CD8+CD11a+ leukocytes was increased, as was the number of molecules of CD11a on these cells; both measures returned to baseline by 180 min after pain induction. There were no changes in other leukocyte subsets.

As anticipated, plasma EPI was significantly elevated by electrical stimulation. Lack of significant changes in pulse rate, mean arterial blood pressure, cortisol, or NE was likely due to the experimental and relatively mild nature of the pain, in that the mean SF-MPQ total and stress VAS scores recorded during stimulation were in the lower third of their respective ranges. Supporting this interpretation, the elevation in EPI and the non-significant increase in NE were well below the median point of their respective physiologic ranges [11].

The number of CD8+CD11a+ cells and the mean number of CD11a molecules on those cells was increased during nociception. Moreover, stress VAS scores were positively correlated with the number of CD8+CD11a+ cells. Elevations in EPI that occurred following the nociceptive stress were not correlated with changes in CD11a expression, in contrast with prior findings that β-agonist receptor activation is associated with CAM expression on leukocytes [8], and may be due to a number of factors, including the limited sample size and mild nature of the adrenergic stimulus. CAM expression may have been mediated by the interaction of lymphocytes with catecholamine neurotransmitter molecules arising from neural impulses transmitted directly to lymphoid organs, but not reflected in correlated plasma levels. Alternatively, the proinflammatory cytokine interleukin-1 is known to exist within storage vesicles in immune cells, and the nociceptive stimulus may have elicited release of these molecules, demonstrated to stimulate leukocyte CAM expression [12]. Cortisol-immune cell interactions secondary to mild hypercortisolism is another plausible mechanism to explain the observed changes in CAM expression. Cortisol molecules may act alone in this role, however, it has been suggested that a concomitant increase in cortisol does not alter catecholamine-related immune cell activation, and may in fact enhance it [13]. The absence of a comparable control group receiving no painful stimulus makes it impossible to assess the contributions of diurnal periodicity and the painful stimulus to alterations in the rate of cortisol reduction (slope of the curve) over the four data collection points in the protocol.

It is not entirely clear why CAM expression was enhanced on CD8+ cells, but not CD4+ cells or monocytes. Though this is the first study of electrical stimulation-related integrin expression, in previous studies of exercise and psychological stress, CD11a expression was found to be elevated in mixed lymphocytes, but not in monocytes or granulocytes [8, 14]. CD8+ lymphocytes are more responsive than CD4+ cells or monocytes to β-adrenergic receptor-mediated migration into the circulation, and similar or related pathways may drive CAM expression [13]. Further, the transient nature of increased CAM expression in the current study (fig. 1) is consistent with the
rapid decline in sympathetic neural impulses, and circulating catecholamine molecules following pain cessation, due to reuptake and metabolism [15].

A number of limitations existed in this study. The focus on integrin CAMs limited this study from investigating potentially pain-responsive inflammatory molecules such as other CAMs (e.g. selectins). Likewise, the study did not investigate proinflammatory cytokine responses though we have evidence that the plasma concentration of molecules of IL-1 receptor antagonists is elevated by acute nociceptive pain [unpubl. data]. The sample size was small, and the study was not powered to examine gender effects. Female hormone plasma levels were not assessed, and this may have obscured information regarding gender-related pain responses. Though separate pain and stress instruments were employed, these two simultaneous and related experiences may have been confounded and difficult for subjects to differentiate, thus obscuring the ability of the study to accurately link the independent and dependant variables. The use of electrical stimulation of the earlobe as the sole pain model in the experiment may have limited or affected the results of the study in unknown ways due to the unique characteristics (nonselective stimulation of all fiber types) of this stimulus modality. Finally, as previously noted, the study lacked a separate control group, precluding the ability to rule out the effects of diurnal variation and testing on cortisol-induced CAM expression. Of note, there is evidence that CD11b (an integrin molecule similar to CD11a) expression does not change in persons during a resting condition [14].

Utilizing a novel pain model, we have focused here primarily on the effects of acute, nociceptive pain on integrin CAM expression in healthy controls. Though the nociceptive stimulus and elevation in the plasma EPI level were relatively mild, increased integrin expression was observed during painful stress, in accordance with responses to other adrenergic stressors [8, 13]. Integrin molecules play a crucial role in the final step of immune cell trafficking, in which firm leukocyte-endothelium adhesion develops, followed by extravasation of leukocytes into inflammatory lesions [16]. Existing evidence links monocyte and lymphocyte infiltration in the context of elevated integrin expression to multiple inflammatory diseases including atherosclerosis [17]. Thus increased expression of CD11a molecules, related to neural pain impulses and stress perception, have implications for symptom exacerbation in patient populations with inflammatory disease syndromes and further support effective diagnosis and treatment of pain in such disorders.

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