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Research article

INCREASED PRESSURE STIMULATES ABERRANT DENDRITIC CELL MATURATION

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Abstract: Patients with malignancy typically exhibit abnormal dendritic cell profiles. Interstitial tumor pressure is increased 20-50mmHg over that in normal tissue. We hypothesized that elevated pressure in the tumor microenvironment may influence dendritic cell (DC) phenotype and function. Monocyte-derived immature and mature DC isolated from healthy human donors were exposed to either ambient or 40 mmHg increased pressure at 37°C for 12 hours, then assessed for expression of CD80, CD86, CD83, CD40, MHC-I and MHC-II. IL-12 production and phagocytosis of CFSE-labeled tumor lysate were assessed in parallel. Elevated pressure significantly increased expression of all co-stimulatory and MHC molecules on mature DC. Immature DC significantly increased expression of CD80, CD86, CD83 and MHC-II, but not MHC-I and CD40, versus ambient pressure controls. Pressure-treated immature DC phenotypically resembled mature DC controls, but produced low IL-12. Phenotypic maturation correlated with decreased phagocytic capacity. These results suggest increased extracellular pressure may cause aberrant DC maturation and impair tumor immunosurveillance.

Key words: Pressure, Dendritic cell, Maturation, Cancer, Immunosurveillance

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Abbreviations used: DC – dendritic cell; GM-CSF – granulocyte-macrophage colony-stimulating factor; IL-4 – interleukin-4; IL-12 – interleukin-12; LPS – lipopolysaccharide; MHC – major histocompatibility complex

INTRODUCTION

The immune system plays a primary role in the maintenance of tissue homeostasis through the identification and elimination of damaged or compromised cells. Regulated induction of immune cells is crucial for protection from foreign pathogens, efficient wound healing, and adequate tumor surveillance [1]. Impairment of the host immune response due to functionally defective antigen-presenting cells (APCs) is thought to be one of the key mechanisms by which tumors escape immune control, as well as a major factor limiting the success of cancer immunotherapy [2]. Dendritic cells (DC) are considered to be the most potent of APCs, and are critical for the induction of effective antigen-specific T-cell responses [3]. Although various reports have documented abnormalities in the dendritic cell profiles of patients with malignancy, the mechanisms underlying such irregularities are poorly understood [4-6].

The functional characteristics of dendritic cells depend upon their state of maturation. Immature dendritic cells are highly efficient at phagocytosis and antigen processing in the peripheral blood and tissues, but express relatively low levels of major histocompatibility complex (MHC) and co-stimulatory molecules necessary for T-cell stimulation. Following antigen capture, dendritic cells differentiate toward a mature phenotype, typically noted by an increase in expression of MHC and co-stimulatory molecules, cytokine secretion, reduced phagocytic ability, and migration to regional lymph nodes where they present antigens to naïve T-cells [7, 8]. Maladaptive variations in this process may result in a weakened immune response, generation of antigen tolerance, autoimmunity, or dendritic cell apoptosis [9-12].

Tumors experience elevated interstitial fluid pressures of 20-50 mmHg over pressures found in normal adjacent tissues [13-16]. Twenty four hour exposure to increased extracellular pressure has previously been shown to stimulate inflammatory cytokine production and enhanced phagocytosis of serum-opsonized latex beads in PMA-differentiated THP-1 macrophages [17]. We therefore hypothesized that human dendritic cell function and phenotype may be similarly sensitive to pressure fluctuations found in the tumor microenvironment. Although phenotypic changes associated with DC maturation become apparent in as few as 4-8 hours following activation, we chose to assess DCs at a 12 hour time point to maximize detection of pressure-mediated effects while minimizing confounding time-dependent responses associated with some markers [18, 19].

To test this hypothesis, DCs were generated by granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 treatment of monocytes isolated from healthy human donors. We assessed the effect of pressure on DC phenotype by evaluating the expression of surface markers CD80, CD86, CD83, CD40, MHC class I and MHC class II on immature and lipopolysaccharide (LPS)-matured DC exposed overnight to either ambient or 40 mmHg increased pressure.

Pressure-induced functional changes in DC IL-12 production and phagocytosis of tumor cell lysate were assessed in parallel studies.

MATERIALS AND METHODS

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphocyte Separation Medium (Mediatech, Herndon, VA). DCs were derived from plastic-adherent monocytes after 7 days in RPMI 1640 medium with 10% autologous serum, supplemented with GM-CSF (1000 U/ml; Amgen/Immunex, Thousand Oaks, CA) and IL-4 (500 U/ml; R&D Systems, Minneapolis, MN). On day 6, half of the DCs were treated overnight with 1 μg/ml of LPS (E. coli serotype 026:B6, Sigma) in order to generate age-matched mature and immature DC populations. PBMC isolation from healthy human donors was approved by the Wayne State University Human Investigation Committee.

Pressure application

Pressure was controlled using an airtight Lucite box with an inlet valve for gas application and an outlet valve connected to a manometer [20, 21]. The box was prewarmed to 37°C to prevent internal temperature and pressure fluctuations. Temperature was maintained at 37 ± 2°C and pressure at 40 ± 1.5 mmHg throughout the duration of the 12 hour experiment. Control cell populations were maintained at ambient pressure in the same incubator.

DC staining and flow cytometric analysis

Following exposure to experimental conditions, dendritic cells were washed twice with phosphate buffered saline (PBS) and fixed with 1% paraformaldehyde. After fixation, cells were washed twice with staining buffer containing 0.2% BSA and 0.02% sodium azide in PBS and incubated with anti-CD80 (PE-L307.4), anti-CD86 (PE-IT2.2), anti-CD83 (PE-HB15a), anti-HLA-DR (FITC-G46-6), anti-HLA-class I (FITC-BB7.2) and anti-CD40 antibodies (BD Pharmingen, San Diego, CA) for 30 minutes at 4°C. DCs were then washed twice with staining buffer and analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and WinMDI software. Flow cytometric analysis was performed strictly on cells within a standard DC gate, based on size and granularity.

Measurement of IL-12 by enzyme-linked immune absorbent assay (ELISA)

Following a 12 hour exposure to increased or ambient pressure conditions, supernatant was collected from DCs seeded at 2.5 x 10^5 cells/ml in RPMI-1640 medium supplemented with 10% autologous serum. Concentrations of immunoreactive IL-12 p40 in supernatants were determined by sandwich ELISA according to the manufacturer's recommendations using paired antibodies obtained from PharMingen (BD Biosciences, San Diego, CA).

Phagocytosis assay

Tumor cell lysate was prepared from SW620 human colon cancer cells incubated at 37°C for 10 minutes with 12.5 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR) in PBS containing 0.1% BSA. Labeled-cells were washed twice with PBS/0.1% BSA in order to remove all excess CFSE and prevent any non-specific DC staining. The tumor cells were then lysed by repeated freeze-thaw cycles. Following the 12 hour incubation of DC under ambient or increased pressure conditions, 50 μl of tumor cell lysate was diluted 1:5 in wells containing 5.0 x 10^4 DCs each. The DCs were incubated with the CFSE-labeled lysate under the same experimental conditions for an additional 2 hours. DCs were then washed twice and stained with anti-CD11c mAb (BD Pharmingen) for 30 minutes at 4°C. DC phagocytosis was assessed by flow cytometric analysis of double-positive stained cells.

Statistical analysis

Statistical analysis was done using SigmaStat software (SPSS, Inc., Chicago, IL). Student t tests or paired t tests were employed as appropriate. A 95% confidence interval was set a priori as the desired level of statistical significance.

RESULTS

Increased extracellular pressure enhances co-stimulatory and MHC molecule expression on mature dendritic cells

Naïve T-cell priming requires antigen presentation by DC in the context of appropriate MHC molecules with adequate co-stimulation from adjacent surface receptors. Mature DC express high levels of MHC class II, CD80, CD86, CD83, and CD40 in order to aid in this process [3]. Under ambient pressure, baseline expression of all surface markers other than MHC class I were significantly elevated (Fig. 1; bar 3 vs. 1; $n = 3$; $p < 0.01$) in LPS-matured DC compared with untreated (immature) DC, consistent with a mature phenotype. Following exposure to elevated pressure, mature DC expression of CD80 and CD86 was further increased by 44 ± 7% ($n = 3$; $p < 0.04$) and 35 ± 4% ($n = 3$; $p < 0.03$), respectively (Fig. 1A and B). Likewise, mature DC expression of CD40 was enhanced by 20 ± 6% (Fig. 1D; $n = 3$; $p < 0.05$). Surface expression of CD83 was elevated by 10 ± 2%. However, differences were not significant (Fig. 1C; $n = 3$). Pressure-treatment of mature DC also increased MHC class I expression by 72 ± 8% (Fig. 1E; $n = 3$; $p < 0.001$) and further enhanced MHC class II expression by 34 ± 9% (Fig. 1F; $n = 3$; $p = 0.04$). These results suggest that increased extracellular pressure enhances LPS-induced DC maturation and may be demonstrative of an adaptive response heightening DC immunogenicity under increased lymphatic or inflammatory pressures.

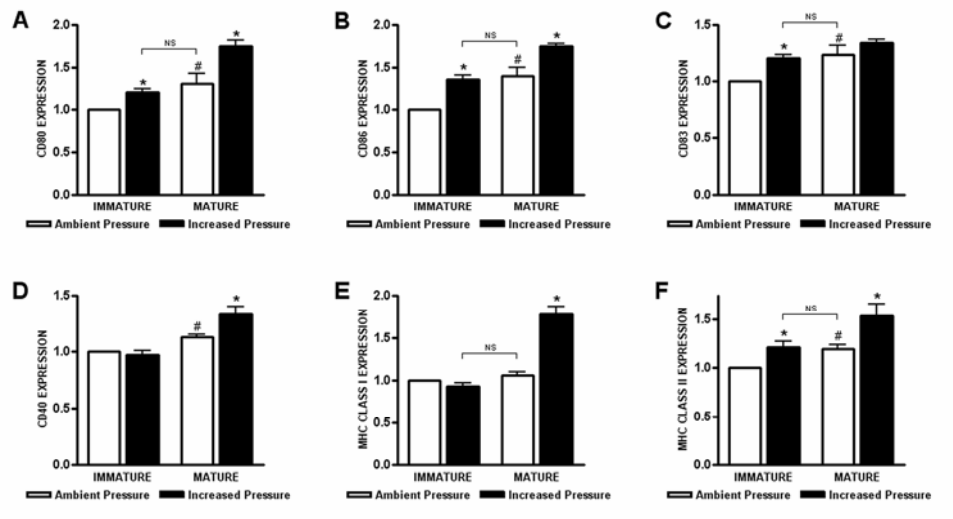

Fig. 1. Effect of increased pressure on DC surface expression of co-stimulatory and MHC molecules. Immature and mature DC surface expression of (A) CD80, (B) CD86, (C) CD83, (D) CD40, (E) MHC class I and (F) MHC class II were compared under ambient (*open bars*) and 40 mmHg increased pressure (*closed bars*) conditions. Expression data is representative of the mean fluorescence intensity of each marker normalized against its respective immature DC ambient pressure control and expressed as mean ± SEM. $^{*}P < 0.05$ comparison of pressure groups vs. respective ambient controls; $^{\#}P < 0.05$ comparison of immature vs. mature DC baseline values; n = 3.

Increased extracellular pressure stimulates aberrant maturation of immature dendritic cells

MHC molecules loaded with autologous peptides in the vicinity of high levels of CD80 and CD86 may induce aberrant T cell responses. Thus, DC maturation and the upregulation of co-stimulatory molecules is typically thought to be a regulated process requiring activation by foreign antigen [3]. Interestingly, 12 hour exposure of immature DC to 40 mmHg increased pressure stimulated a 20 ± 4% (n = 3; p = 0.008) and 36 ± 4% (n = 3; p < 0.001) increase in CD80 and CD86 expression (Fig. 1A and B). CD83 and MHC class II expression were also increased by 20 ± 3% (n = 3; p < 0.002) and 21 ± 6% (n = 3; p < 0.03), respectively (Fig. 1C and F). However, pressure-treated immature DC displayed no change in CD40 expression (Fig. 1D; n = 3; differences not significant) and an approximate 7% decrease in MHC class I expression (Fig. 1E; n = 3; differences not significant). Pressure-induced surface expression of CD80, CD86, CD83, and MHC class II on immature DC was not significantly different from that of LPS-matured DC maintained under ambient pressure. These results are consistent with the concept that elevated pressure induces phenotypic maturation of DC in the absence of activation by foreign antigen.

Increased pressure significantly enhances IL-12 production in mature, but not immature DC

IL-12 produced by DCs during antigen presentation promotes T cell differentiation and results in the enhancement of cell mediated immunity. Likewise, increased IL-12 production is typically associated with DC maturation. Following exposure to ambient or increased pressure conditions, supernatant from immature and mature DC was collected and assessed for IL-12 production by ELISA (Fig. 2). LPS-matured DC kept under ambient conditions released significantly greater amounts of IL-12 (1182 ± 77 pg/ml; n = 3; $p < 0.004$) compared with immature DC (70 ± 12 pg/ml; n = 3) incubated under similar conditions. Exposure to elevated pressure increased mean IL-12 release by 105% (2429 ± 203 pg/ml; n = 3; $p < 0.001$) in mature DC whereas IL-12 production in pressure-treated immature DC was only modestly affected (88 ± 11 pg/ml; n = 3; differences not significant) despite a mature phenotype.

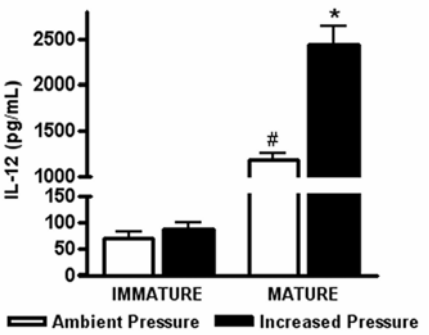

Fig. 2. Effect of increased pressure on DC IL-12 production. Supernatants from immature and mature DCs were harvested following exposure to either ambient (*open bars*) or 40 mmHg increased pressure (*closed bars*) conditions. Levels of IL-12 p40 in cell supernatants were measured by ELISA. Data are represented as mean ± SEM. $^{*}P < 0.001$ comparison of pressure groups vs. respective ambient controls; $^{\#}P < 0.001$ comparison of immature vs. mature DC baseline values; n = 3.

Pressure-induced phenotypic maturation of DC correlates with reduced phagocytic capacity

As part of their normal function, DCs non-discriminatorily sample their tissue microenvironment, phagocytizing both self and non-self constituents for clearance, degradation, and presentation [22]. A reduced capacity to phagocytose particulate antigens is typically believed to accompany DC maturation [23]. We next investigated whether pressure-induced phenotypic changes in DC surface molecule expression also corresponded with functional changes in phagocytic capacity. Following exposure to ambient or increased pressure, immature and mature DCs were incubated with CFSE-labeled tumor cell lysate for an additional 2 hours under their respective experimental conditions. Phagocytic capacity was determined by flow cytometric analysis of

DC fluorescence (Fig. 3). As expected, phagocytosis of tumor cell lysate was decreased by 17 ± 3% (n = 3; p < 0.02) in LPS-matured DC versus immature DC kept at ambient pressure. Consistent with our previous data, exposure to increased extracellular pressure reduced immature DC phagocytosis by 18 ± 4% (n = 3; p = 0.01) and further decreased LPS-matured DC phagocytosis on average by 6% (n = 3), although differences were not significant. These results further support a negative role for pressure-stimulated DC maturation in maintaining immune-mediated tumor control.

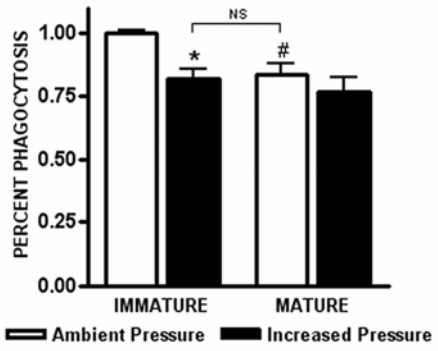

Fig. 3. Effect of increased pressure on DC phagocytic capacity. Immature and mature DCs were incubated with CFSE-labeled tumor cell lysate for two hours following exposure to either ambient (*open bars*) or 40 mmHg increased pressure (*closed bars*) conditions. Phagocytosis data is representative of the mean fluorescence intensity of CD11c-CFSE double-positive cells normalized against the immature DC ambient pressure control and expressed as mean ± SEM. $*P < 0.05$; n = 3.

DISCUSSION

Overcoming tumor-mediated suppression of dendritic cell activity is essential for successful immunotherapy against cancer. Regardless of sufficient recruitment and tumor infiltration, functionally aberrant DCs have been observed with multiple human malignancies [24-26]. This supports the idea that factors in the immediate tumor microenvironment are likely responsible for DC impairment. Interstitial fluid pressures within the tumor bed may reach as much as 50 mm Hg over that in surrounding tissue [15]. Our current data suggest that DC exposure to increased extracellular pressure stimulates maturation and may promote a functionally aberrant phenotype through the altered expression of co-stimulatory molecules, low IL-12 production, and impaired uptake of tumor-derived antigens.

Increased extracellular pressure failed to stimulate CD40 expression in concordance with other co-stimulatory molecules on immature DC. DC CD40 interaction with CD40L on T cells acts to support T cell activation through upregulation of its IL-2 receptor and is essential for production of IL-12 by

DCs [27]. CD40 ligation also serves as a prosurvival signal for DC and is required for their full functional maturation [27]. Stimulation of DCs by CD40L leads to an increase in expression of the anti-apoptotic protein Bcl-X_L and was demonstrated to increase the resistance of DCs to apoptosis in patients with prostate cancer [28]. Low expression of CD40 on pressure-matured DCs suggests this population of cells may be poorly suited to generate an adequate anti-tumor T cell response as well as being overtly sensitive to apoptosis.

In vivo studies have demonstrated that DC IL-12 production is also necessary for adequate T cell stimulation [29]. Mature DCs secreting high levels of IL-12 exhibit an increased capacity to sensitize more avid $CD8^+$ T cell responses towards tumor-associated antigens [30]. Pressure-matured DCs displayed extremely low IL-12 production in comparison with LPS-matured DCs. Low IL-12 secretion by pressure-matured DCs may further detract from their ability to prime a sufficient immune response. Although increased pressure appears to enhance IL-12 production in LPS-matured DC, it is important to note that overexpression of IL-12 and CD40 are also associated with a variety of chronic inflammatory conditions [31, 32]. Thus, it is difficult to differentiate between what is a beneficial adaptive response and what may be pathogenic.

While several recent studies have reported cancer patients to exhibit an overall reduction in number of circulating DCs and an increase in the relative amounts of immature DCs, this work has focused on the effects of tumor-derived soluble factors on the abnormal differentiation of myeloid cells, and may be tumor type-specific [33, 34]. Tumor secretion of macrophage colony-stimulating factor (M-CSF) by renal-cell-carcinomas was reported to impair DC differentiation [35]. Also, tumor-mediated production of IL-6 has been shown to inhibit functional differentiation of DCs in patients with multiple myeloma [36]. However, while these studies offer alternate explanations to the root cause of DC dysfunction in cancer, it is important to note that our current investigation assesses the effects of increased pressure on already differentiated DCs.

Although further experiments assessing the effects of increased pressure on DC capacity to prime T cell responses are still required, our current data suggests that elevated interstitial fluid pressure within the tumor microenvironment serves as an activating maturation stimulus for human monocyte-derived DCs. Pressure-induced phenotypic changes in mature DCs may reflect an adaptive response enhancing DC immunogenicity under increased lymphatic pressure. However, the same phenomenon imposed upon immature DCs in the tumor microenvironment may generate functionally aberrant mature DCs ill-equipped to generate an adequate anti-tumor immune response. Elucidation of the intracellular signals modulating this effect may identify novel targets for immunotherapeutic intervention.

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