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NaCl Potentiates Human Fibrocyte Differentiation

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Abstract

Excessive NaCl intake is associated with a variety of fibrosing diseases such as renal and cardiac fibrosis. This association has been attributed to increased blood pressure as the result of high NaCl intake. However, studies in patients with high NaCl intake and fibrosis reveal a connection between NaCl intake and fibrosis that is independent of blood pressure. We find that increasing the extracellular concentration of NaCl to levels that may occur in human blood after high-salt intake can potentiate, in serum-free culture conditions, the differentiation of freshly-isolated human monocytes into fibroblast-like cells called fibrocytes. NaCl affects the monocytes directly during their adhesion. Potassium chloride and sodium nitrate also potentiate fibrocyte differentiation. The plasma protein Serum Amyloid P (SAP) inhibits fibrocyte differentiation. High levels of extracellular NaCl change the SAP Hill coefficient from 1.7 to 0.8, and cause a four-fold increase in the concentration of SAP needed to inhibit fibrocyte differentiation by 95%. Together, our data suggest that NaCl potentiates fibrocyte differentiation. NaCl-increased fibrocyte differentiation may thus contribute to NaCl-increased renal and cardiac fibrosis.

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Introduction

Fibrosing diseases such as pulmonary fibrosis, congestive heart disease, and renal fibrosis involve the formation of unwanted scar tissue in internal organs [1,2]. The scar tissue in fibrosis leads to organ malfunction and subsequent organ failure [1]. Fibrosis is associated with approximately 45% of deaths in the U.S [1]. Fibrosis involves infiltration of blood leukocytes into the affected organs, activation and/or appearance of fibroblast-like cells, tissue remodeling, and deposition of extracellular matrix proteins such as collagen [3–8]. Fibrosis can be caused by factors such as environmental toxins, or aberrant healing events [6].

Fibrocytes are CD45^{+}, collagen I^{+} fibroblast-like cells that have been implicated in scar tissue formation and fibrosis [3,5,6,8,9]. These cells share similarities with both blood leukocytes and tissue resident cells [7,9]. Fibrocytes, depending on the environmental cues, can express extracellular proteases, or matrix proteins such as collagen [6,10]. Fibrocytes differentiate from CD14^{+} monocytes [8]. Following their recruitment to a specific tissue, monocytes can differentiate into macrophages, dendritic cells, or fibrocytes [6,11,12]. Serum Amyloid P (SAP), a pentameric protein from the pentraxin family of proteins, interacts with Fc receptors on monocytes to inhibit fibrocyte differentiation [13–16].

Monocytes leave the bone marrow and travel through the blood vessels until they are recruited into a specific tissue in response to chemokines. Afterward, they mature and differentiate under the influence of signaling molecules such as M-CSF, GM-CSF, IL-4, IL-13, and TGF-β1 [17–20]. One component of monocyte activation and differentiation is a group of receptors belonging to the integrin family of proteins [21,22]. Integrins are composed of α and β subunits [23]. These proteins aid in monocyte adhesion to components of extracellular matrices and are central to inflammation, immunity, and homeostasis [24]. The adhesive properties of integrins contribute to different patterns of monocyte differentiation under various conditions [21,22,25].

Much remains to be understood about the mechanisms which trigger fibrosis and fibrocyte differentiation. Congestive heart disease and renal fibrosis have previously been linked to high NaCl intake in both humans and various animal models, but the exact mechanism underlying this connection is unknown [2,26–30]. Sodium and chloride ions contribute to the maintenance of electrical gradients across the membrane of many cells. In addition, sodium and chloride ions are used to absorb nutrients such as amino acids in the intestine, and regulate blood pressure and volume [26,31,32]. The latter function of sodium and chloride ions has been the focus of many studies, since abnormalities in blood pressure have been associated with stroke, cardiac fibrosis, and renal fibrosis [27].

It is generally accepted that there is a direct correlation between high salt intake and high blood pressure which in turn increases the chances of heart disease, stroke and renal failure [27]. Until recently, it was believed that high blood pressure is the main contributor to heart disease and renal failure. However, animal and clinical studies have shown the deleterious effects (i.e. stroke, cardiac and renal fibrosis) of salt in the absence of increased blood pressure [26,29,33,34]. For instance, Wistar or Wistar-Kyoto rats exposed to salt overload develop cardiac, vascular, and renal fibrosis without exhibiting a significant increase in blood pressure [28,29]. In addition, a connection between salt intake and fibrosis that is independent of high blood pressure has been previously established [26,35,36].

A variety of studies indicate that different ions can modulate immune cell function. For instance, potassium channels and potassium transport affect the integrin-dependent activation of the monocytic-derived cell line, THP-1 [37]. Altering cation transport in murine erythroleukemia cells can induce differentiation [38].
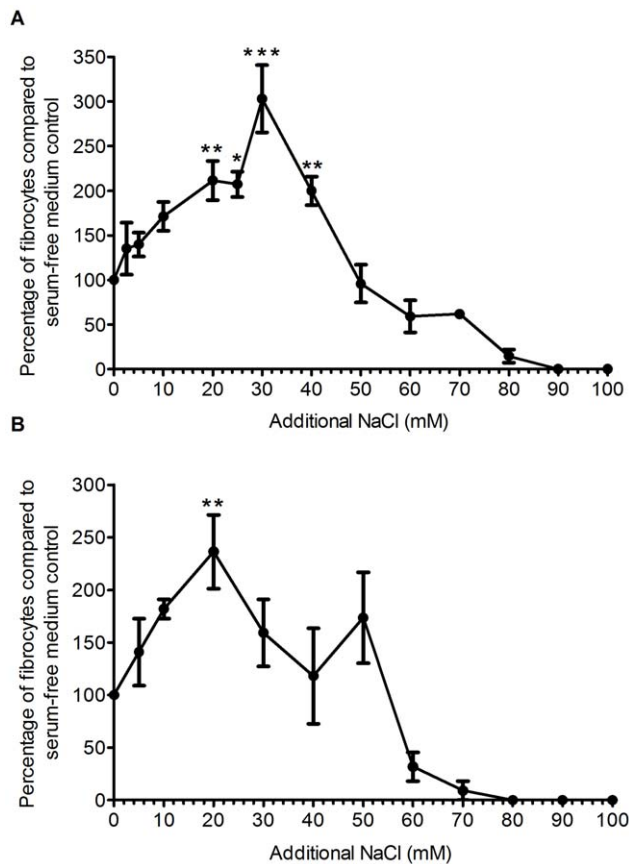
Figure 1. Addition of NaCl to Fibrolife or RPMI based-medium increases fibrocyte differentiation. Human PBMCs were cultured for 5 days in serum-free medium in the presence of the indicated concentrations of additional NaCl in either Fibrolife (A) or RPMI (B) medium. Results are expressed as mean ± SEM, n = 8. * indicates statistical significance with p<0.05, ** indicates P<0.01, and *** indicates p<0.001 by ANOVA compared to the no additional NaCl control.
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This, combined with the increased instances of fibrosing diseases in patients with high dietary salt intake, caused us to investigate the role of NaCl in the activation of monocytes and their subsequent differentiation into fibrocytes. Our results suggest that high concentrations of NaCl potentiate fibrocyte formation.

Results

Additional NaCl can potentiate fibrocyte formation without influencing cell viability

Since fibrocytes are implicated in fibrosing diseases, and NaCl may promote fibrosis, we examined the hypothesis that NaCl might affect fibrocyte differentiation. Using PBMCs from a variety of donors, we observed 600 to 2800 fibrocytes per 10^5 PBMCs in both Fibrolife and RPMI 1640 medium. For all donors, additional NaCl significantly potentiated fibrocyte differentiation, when compared to the control with no added NaCl (Figure 1A and B). In Fibrolife medium, NaCl caused a maximum potentiation of fibrocyte differentiation at 30 mM additional NaCl. The [Na⁺] and [Cl⁻] concentrations in Fibrolife medium are 126 mM and 119 mM, respectively. At 30 mM additional NaCl, both the sodium and the chloride concentration of Fibrolife medium ([Na⁺] = 156 mM, [Cl⁻] = 144 mM) are in excess when compared to
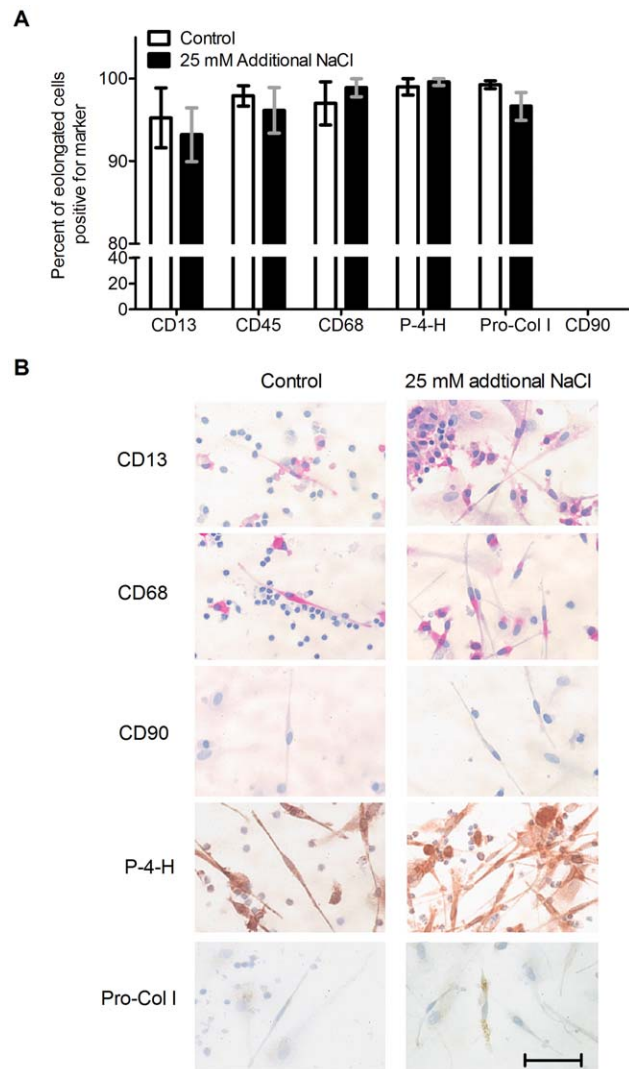

Figure 2. The elongated cells are fibrocytes. PBMCs were cultured in Fibrolife medium for five days in the presence or absence of 25 mM additional NaCl. Cells were fixed and stained for the indicated markers. (A) The elongated spindle-shaped cells in the wells stained positive for CD13, CD45, CD68, Prolyl 4-hydroxylase (P-4-H), Pro-Collagen I (Pro-Col I) and had no apparent staining for the fibroblast marker CD90. Results are expressed as mean ± SEM, n = 3. (B) Cells were stained with the indicated antibodies. Positive staining was identified by red for alkaline phosphatase staining and brown for peroxidase staining. Cells were counterstained blue with hematoxylin to identify nuclei. Size bar is 50 μm.
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human blood ([Na⁺] = 137 mM, [Cl⁺] = 102 mM) [39,40]. The [Na⁺] and [Cl⁻] concentrations in RPMI 1640 medium are 134 mM and 107 mM, respectively. In RPMI 1640 medium, NaCl caused a maximum potentiation of fibrocyte differentiation at 20 mM additional NaCl, making [Na⁺] = 154 mM, and [Cl⁻] = 127 mM, again in excess when compared to human blood.

To verify that the NaCl-induced spindle-shaped cells are fibrocytes, we stained PBMCs after 5 days of incubation with or without 25 mM additional NaCl in Fibrolife for fibrocyte markers. In the presence or absence of added NaCl, the elongated cells were positive for the fibrocyte markers CD13, CD45, CD68, pro-collagen I, and prolyl 4-hydroxylase, and showed no observable
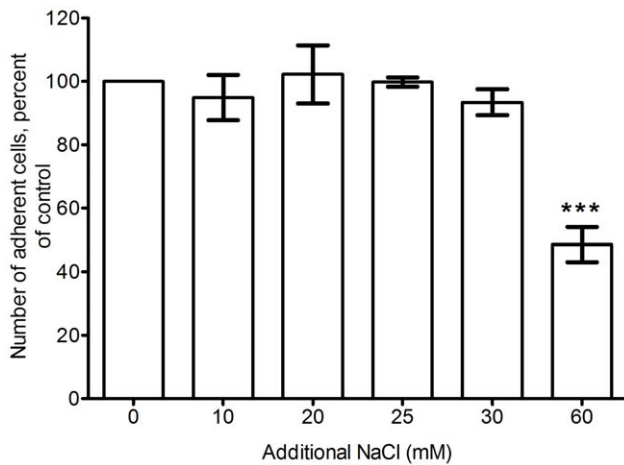
Figure 3. The effect of NaCl on adherent cells after 5 days. PBMCs were incubated with the indicated concentrations of added NaCl, and the number of adherent cells was counted after 5 days. Counts were then normalized to the control with no added NaCl (0). Values are mean ± SEM, n = 3. *** indicates statistical significance by ANOVA compared to the control (p<0.001). doi:10.1371/journal.pone.0045674.g003
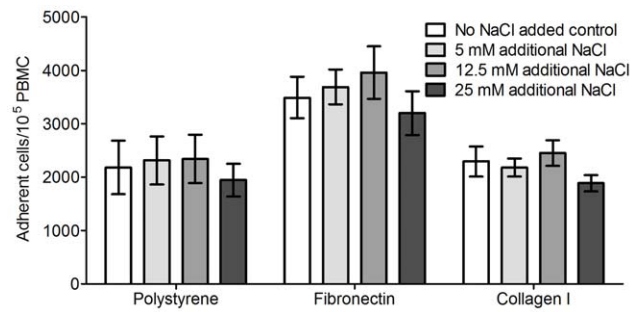

Figure 4. NaCl does not influence the adhesion of PBMCs to plastic, plasma fibronectin or collagen I. PBMCs were incubated on uncoated polystyrene plates, fibronectin coated plates, or bovine collagen I coated plates for one hour in the indicated concentrations of additional NaCl. The non-adhered cells were then rinsed off and the number of adherent cells were counted. Values are mean ± SEM, n = 4. doi:10.1371/journal.pone.0045674.g004

staining for the fibroblast marker CD90, indicating that the elongated cells are fibrocytes, and that NaCl thus is increasing the number of fibrocytes (Figure 2).

An increase in the total number of adherent cells in our assay plates could account for the increased fibrocyte count. To address this possibility, we counted the number of adherent cells after 5 days of incubation with additional NaCl. At 30 mM additional NaCl and below, we observed no significant change in the number of adherent cells as the result of additional NaCl (Figure 3). The addition of 60 mM additional NaCl did however decrease the number of adherent cells, indicating reduced cell viability.

25 mM additional NaCl does not influence PBMC adhesion

Fibrocytes differentiate from $CD14^+$ monocytes [6,8]. Monocytes are activated in response to adhesion to extracellular matrix proteins as well as tissue culture plates [11,12]. To understand the mechanism behind the potentiation of fibrocyte formation by NaCl, we investigated the influence of NaCl on the initial adhesion of PBMCs to plates, since such changes could influence PBMC activation and therefore differentiation. PBMC adhesion to collagen I-coated, fibronectin-coated, or un-coated tissue culture plates was not influenced by increasing NaCl concentrations (Figure 4). However, as previously observed, more PBMCs adhered to fibronectin-coated plates than un-coated plates ([41] and Figure 4). These results suggest that the observed increase in fibrocyte differentiation is not caused by changes in the ability of PBMCs to initially adhere to extracellular matrix proteins or tissue culture plates.

Monocyte to fibrocyte differentiation is potentiated by additional NaCl

The NaCl-induced increase in fibrocyte formation from PBMCs could be due to either secretion of a fibrocyte stimulating factor by non-monocytic cells, or via direct effect of NaCl on monocytes. To distinguish between these two possibilities, we isolated $CD14^+$ cells and examined their differentiation into fibrocytes in the presence of increasing NaCl concentrations. We observed 1400 to 9200

fibrocytes per 10^5 monocytes from 3 different donors. When fibrocyte counts were compared to the no NaCl added control, fibrocyte differentiation was significantly increased by 20 mM to 30 mM additional NaCl (Figure 5). This suggests that monocytes are directly influenced by the additional NaCl, and not through a secreted factor from CD14-negative cells.

PBMCs and monocytes are influenced during their adhesion by additional NaCl

PBMCs are activated by their adhesion to extracellular matrix proteins and tissue culture plates [42]. To elucidate the mechanism behind the NaCl-induced increase in fibrocyte formation, we incubated PBMCs with additional NaCl before, during, and after their adhesion (Figure 6). We observed that incubation of PBMCs with additional NaCl for one hour during their adhesion potentiated fibrocyte formation (Figure 6). Incubation of PBMCs with additional NaCl before or after their adhesion, or for 30 minutes during their adhesion, had no significant effect on fibrocyte formation (Figure 6). These data suggest that NaCl influences fibrocyte precursors during adhesion.

$CD14^+$ monocytes were also incubated with additional NaCl before, during, and after adhesion. Similar to the results with PBMCs, incubation of monocytes with additional NaCl during their adhesion resulted in increased fibrocyte formation (Figure 7). A 30-minute incubation of monocytes with 25 mM additional NaCl was sufficient for this potentiation.

Since additional NaCl potentiates fibrocyte differentiation by influencing monocytes during their initial adhesion to the plate, we examined the effects of 25 mM additional NaCl on different adhesion molecules. We incubated PBMCs in the presence or absence of 25 mM additional NaCl for 1 hour at $37°C$. Following incubation, the cells were stained for CD11a, CD11b, CD11c, CD11b activation epitope, CD18, CD18 activation epitope, CD31, CD49d, and CD62L. The stained cells were subjected to flow cytometry and the $CD14^+$ monocytes were investigated for any change in the mentioned adhesion molecules. We observed no change in the studied adhesion molecules as the result of additional NaCl (Figure 8).

Sodium nitrate but not sodium gluconate potentiates fibrocyte differentiation

Sodium nitrate and sodium gluconate are two classical sodium chloride substitutes used to probe chloride transport [43]. Nitrate can bind and cross chloride channels [43–46]. Gluconate only
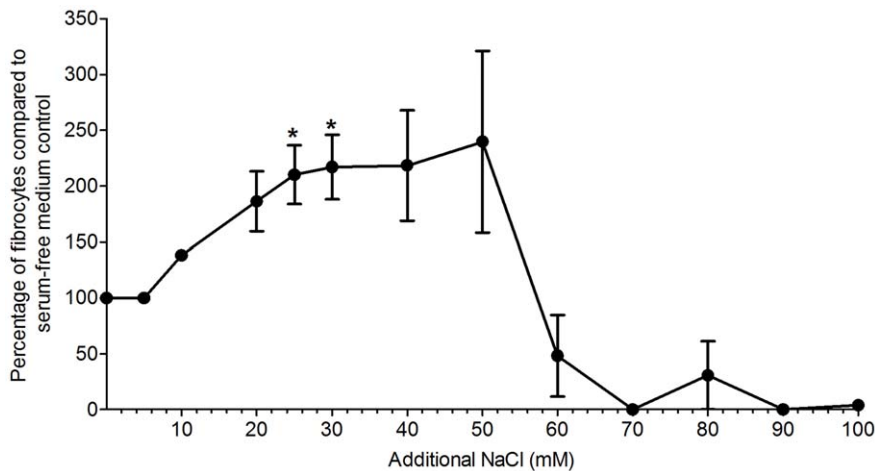
Figure 5. NaCl directly potentiates the differentiation of monocytes into fibrocytes. $CD14^+$ monocytes were incubated with the indicated additional concentrations of NaCl. After 5 days, fibrocytes were counted. Values are mean $\pm$ SEM, n = 3. * indicates p<0.05 compared to control (ANOVA). The absence of error bars indicate that error was smaller than the plot symbol.
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mimics the electrical charge of chloride while being unable to cross the majority of chloride channels [43–47]. Sodium nitrate but not sodium gluconate potentiated fibrocyte differentiation (Figure 9A). The response of PBMCs to sodium nitrate was similar to that of NaCl. This suggests that chloride transport across the membrane is a requirement for potentiation of fibrocyte differentiation by additional NaCl. In addition to sodium nitrate and sodium gluconate, we investigated other compounds such as potassium chloride, rubidium chloride, cesium chloride, choline chloride, tetraethyl ammonium chloride, and monosodium glutamate. Adding 25 mM potassium chloride potentiated fibrocyte differentiation (Figure 9B). Monosodium glutamate had no effect of fibrocyte differentiation but was toxic when added in excess of 50 mM. Choline chloride and rubidium chloride were toxic at concentrations higher than 4 mM, while cesium chloride and tetraethyl ammonium chloride were toxic at 2 mM (data not shown).
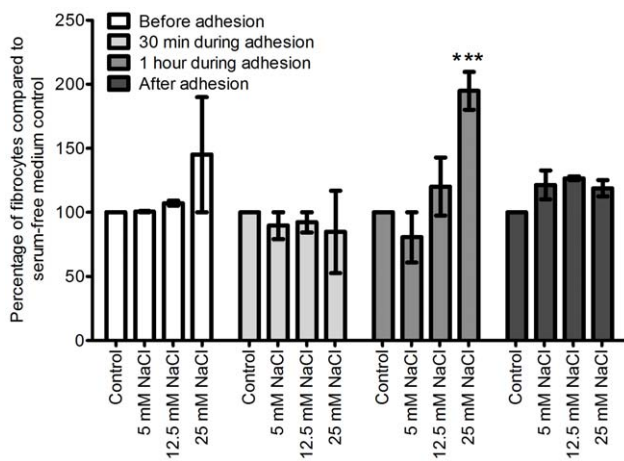
NaCl interferes with the ability of Serum Amyloid P to inhibit fibrocyte differentiation

Human Serum Amyloid P (hSAP) is a pentameric protein secreted from the liver that inhibits fibrocyte differentiation [13,48,49]. The addition of 25 mM NaCl to the medium changed the Hill coefficient for the inhibition of fibrocyte differentiation by hSAP from 1.7 ± 0.1 to 0.7 ± 0.1 (mean $\pm$ SEM, n = 5, p<0.05 by t-test) (Figure 10). In addition, adding 25 mM to the medium significantly inhibited the ability of high concentrations of hSAP to inhibit fibrocyte differentiation (Figure 10). The data indicate that NaCl, in addition to potentiating fibrocyte differentiation, influences how hSAP inhibits fibrocyte differentiation.

Discussion

Monocyte-derived fibrocytes are found in fibrotic lesions, skin wounds, and tumors [3,5–7,50–53]. We have shown here that the

Figure 6. The presence of additional NaCl during PBMC adhesion increases fibrocyte differentiation. PBMCs were incubated with additional NaCl, before, during, before, and after their adhesion to tissue culture treated plates. Values are mean $\pm$ SEM, n = 6. *** indicates p<0.001 compared to control (t-test). The absence of an error bar indicates that the error was smaller than the line thickness.
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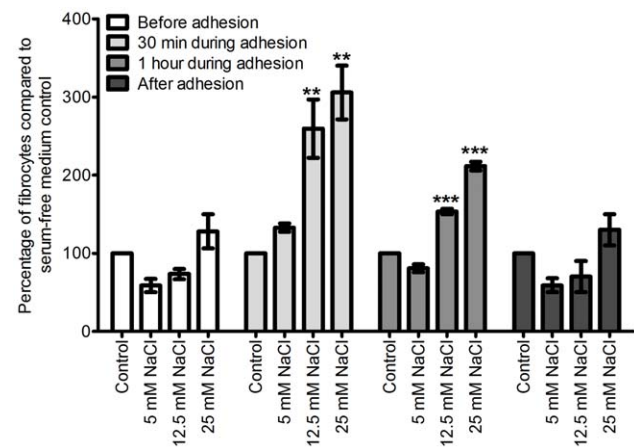

Figure 7. The presence of additional NaCl during $CD14^+$ monocyte adhesion increases fibrocyte formation. Isolated monocytes were incubated with additional NaCl before, during, and after their adhesion to tissue culture treated plates. Values are mean $\pm$ SEM, n = 4, ** indicates p<0.01, and *** indicates p<0.001 compared to control (ANOVA).
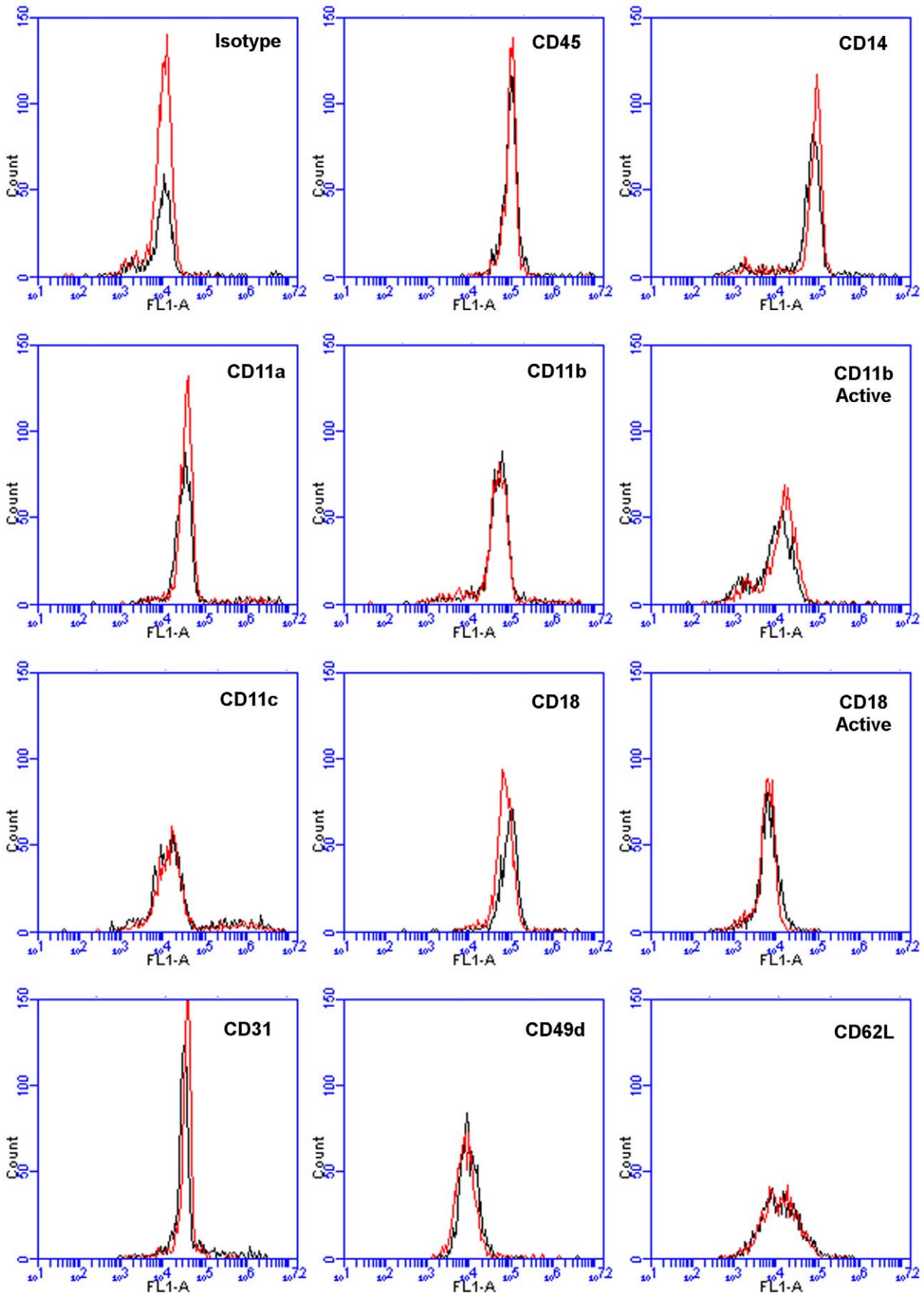doi:10.1371/journal.pone.0045674.g007

Figure 8. Incubation of PBMCs with additional NaCl does not influence the cell-surface levels of several adhesion molecules. PBMCs were incubated in the presence or absence of 25 mM additional NaCl for 1 hour at 37°C and then stained for the indicated adhesion molecules. Monocytes were identified by their forward and side scatter characteristics and the expression of CD14. Flow cytometry plots are representative results from three separate donors. Histograms represent fluorescence intensity of the indicated marker in control cells (Black line) and NaCl treated Cells (Red line).
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differentiation of human monocytes into fibrocytes in serum-free culture is potentiated by NaCl. This increase appears to be a direct effect of NaCl on monocytes. In patients with long-term elevated salt intake, there is a 5% increase in serum NaCl, raising [Na$^+$] from 137 mM to 144 mM [40]. In addition, food intake increases the blood sodium chloride level for ~4 hours [54]. We observed that 155 mM [Na$^+$] and 130 [Cl−] potentiates fibrocyte differentiation, and we hypothesize that this [Na$^+$] and [Cl−] concentration could occur in the serum or tissues of some individuals, at least for a few hours after eating a high-salt meal. However short, this transient increase could be sufficient to increase fibrocyte differentiation.

In animal models and humans, increasing the extracellular osmolarity suppresses neutrophil functions such as cellular cytotoxicity and migration [55,56]. In addition, when tested on PBMCs in vitro, high salt increases the secretion of the pro-inflammatory cytokines IL-8 and TNF-α [57–59]. These studies indicate that extracellular osmolarity can affect cells of the immune system. The increase in fibrocyte differentiation in response to increased NaCl could thus be due to increased osmolarity. However, we observed that adding up to 50 mM sodium gluconate or monosodium glutamate to the medium,

which would also increase osmolarity, did not affect fibrocyte differentiation. Alternatively, an increase in cell proliferation as the result of additional NaCl could explain the increase in fibrocyte count. However, previous studies on the proliferation of fibrocytes and fibrocyte precursors revealed no appreciable proliferation [60]. Thus, the observed increase in fibrocyte differentiation is probably not related to the medium osmolarity or cell proliferation in our in vitro assay and most likely involves a direct effect of NaCl on monocytes.

When monocytes are activated following their recruitment into the tissue and adhesion to extracellular matrices, they undergo changes in gene expression and receptor levels [42,61,62]. Following adhesion, monocytes are activated through a series of intracellular signaling events. We found that NaCl was most effective at increasing fibrocyte differentiation during monocyte adhesion. This was not accompanied by any changes in the expression of adhesion molecules CD11a, CD11b, CD11c, CD18, CD31, CD49d, and CD62L. In agreement with this, the additional NaCl did not influence the adhesive properties of monocytes to plasma fibronectin, collagen I, or un-coated tissue culture plates. Membrane potential and ion transport affect the activation of the monocyte-derived cell line THP-1 [37]. It is thus possible that the effect of NaCl on fibrocyte differentiation is largely due to the increased NaCl affecting membrane potential and ion transport during the adhesion stage of monocyte activation.

Ion channels, especially chloride channels, appear to affect cellular activation and differentiation. In osteoblasts, the chloride channel ClC-3 regulates osteo-differentiation [63]. The development and differentiation of keratinocytes is regulated in part by calcium-gated chloride channels [64]. We observed that sodium nitrate but not sodium gluconate potentiated fibrocyte differentiation. Nitrate can pass through chloride channels, while gluconate cannot [43,46]. Combined with the observation that KCl potentiates fibrocyte differentiation, this suggests that chloride ions might play a role in the NaCl-induced potentiation of fibrocyte differentiation.

Monocyte activation and the subsequent differentiation into fibrocyte is influenced by variety factors such as SAP, M-CSF, GM-CSF, IL-4, IL-13 and, TGF-β1 [8,13,60]. These regulators of the immune system each use distinct and sometimes overlapping signaling transduction pathways making it difficult to identify the exact pathway responsible for the NaCl-induced increase in fibrocyte formation.

Excessive intake of NaCl has been associated with high blood pressure, stroke, heart disease, and kidney disease [26,27,65,66]. The correlation between high salt intake and these diseases has generally been attributed to high blood pressure [27]. However, studies in rats have questioned this correlation [28,29]. In addition, two independent studies on cohorts of patients with high salt intake and fibrosis showed a connection between these two factors that was independent of high blood pressure [35,36]. Here we have shown that fibrocyte differentiation can be potentiated by increasing extracellular NaCl levels in vitro. An intriguing possibility is that the link between high sodium chloride intake and fibrosis involves NaCl potentiating fibrocyte differentiation.
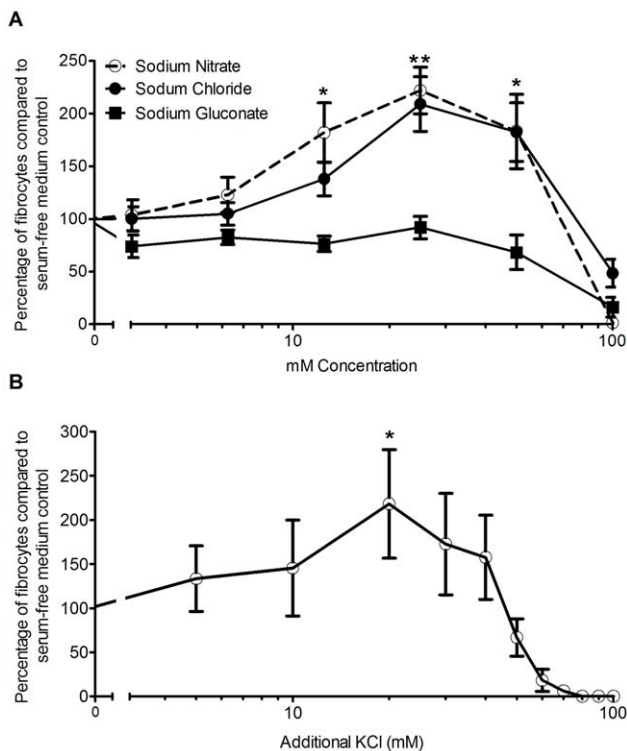

Figure 9. Sodium nitrate and potassium chloride potentiate fibrocyte differentiation. PBMCs were cultured with the indicated concentrations of additional (A) sodium chloride, sodium nitrate, or sodium gluconate, or (B) potassium chloride. Values are mean ± SEM, n = 6. * indicates p<0.05, and ** indicated p<0.01 compared to control by ANOVA.
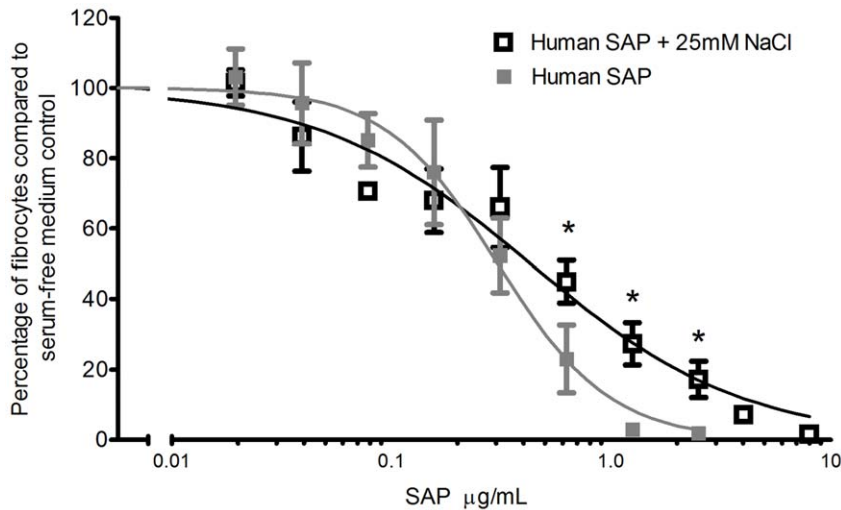doi:10.1371/journal.pone.0045674.g009

Figure 10. NaCl interferes with the ability of hSAP to inhibit fibrocyte differentiation. PBMCs were incubated with or without additional 25 mM NaCl with the indicated concentrations of human SAP (hSAP). After 5 days, fibrocytes were counted. Values are mean $\pm$ SEM, n = 5. * indicates p<0.05 compared to SAP alone control (dashed line) by ANOVA. The absence of error bars indicate that error was smaller than the plot symbol. Lines are sigmoidal dose-response curves with variable Hill coefficient fit to the data.
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In addition to participating in fibrosis, fibrocytes participate in wound healing [6,7,67]. Our observations suggest that having ~155 mM [Na^+] and ~ 130 mM [Cl^-] in wound dressings could not only potentiate fibrocyte differentiation but also influence the ability of hSAP to inhibit fibrocyte differentiation, and thus potentiate wound healing.

Materials and Methods

PBMC and monocyte isolation, cell culture, and fibrocyte differentiation assay

Human blood was collected into heparin tubes (BD Bioscience, San Jose, CA) from adult volunteers who gave written consent and with specific approval from the Texas A&M University human subjects Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway, NJ) and cultured in Fibrolife (LifeLine Cell Technology, Walkersville, MD) medium or where indicated in RPMI 1640 (SigmaAldrich, St. Louis, MO) as described previously [68]. CD14$^+$ monocytes were isolated by negative selection using magnetic Dynabeads (Dynal Biotech, Milwaukee, WI) as described previously [13]. CD14$^+$ monocytes were plated in 96-well plates with 5×10^4 cells in 200 µl per well. After 5 days, the plates were air dried, fixed with methanol and stained using a Hema 3 staining Kit (Thermo Fisher Scientific, Milwaukee, WI) [68]. Fibrocytes were identified and counted based on their elongated spindle-shaped morphology in five different 900 µm-diameter fields of view per well.

Salt Solutions and Serum Amyloid P purification

One molar solutions of sodium chloride, sodium gluconate, sodium nitrate, and other salts were prepared in Fibrolife (LifeLine Cell Technology) basal medium or RPMI-1640 (SigmaAldrich) where indicated. The 1 M solutions were then filter sterilized using Acrodisc 0.2 µm syringe filters (Pall, Port Washington, NY). Prepared solutions were then added to the wells at the indicated concentrations. Human Serum Amyloid P was purified from human serum as described previously [49].

Immunohistochemistry

PBMCs were cultured for 5 days on eight-well glass microscope slides (Thermo Fisher) as described previously [9]. The cells were then air dried before fixation in acetone for 10 minutes. The cells were stained for CD13, CD45, CD68, Pro-collagen I (Developmental Studies Hybridoma Bank, University of Iowa), CD90, and Prolyl 4-hydroxylase as described previously [9].

Adhesion Assay

96-well polystyrene plates (BD Biosciences) were coated with 15 ug/mL of bovine plasma fibronectin (SigmaAldrich) or bovine collagen I (SigmaAldrich) for 1 hour at 37°C. The plate was then washed 3 times with PBS and then blocked with 4% BSA (SigmaAldrich, cat # A3059) in PBS for 1 hour at 37°C. Afterward, the plate was washed with serum-free medium, and peripheral blood mononuclear cells were incubated on the plate for 1 hour at 37°C. The plates were then washed 3 times with PBS and adherent cells were counted in five 900 µm-diameter fields of view per well.

PBMC/monocyte pulse experiment

PBMCs or monocytes were incubated with additional concentrations of NaCl for the indicated times at 37°C to determine the effect of additional of NaCl on fibrocyte differentiation during adhesion. Then, approximately 95% of the medium was gently removed, so as not to disturb the cells, and replaced with fresh medium. The cells were left to differentiate for 5 days. To determine the effect of NaCl on adherent cells, PBMCs or monocytes were incubated in 96-well plates for 1 hour at 37°C, and the medium was then replaced with medium containing additional NaCl. The cells were left for 1 hour at 37°C, and subsequently the medium was replaced with fresh medium with no additional NaCl. These cells were then left to differentiate for 5 days. To elucidate the effect of NaCl on PBMCs or monocytes before their adhesion to the plate, cells were placed in BSA-coated eppendorf tubes for 1 hour in medium with additional NaCl at 4°C. The cells were then collected by centrifugation for 5 minutes at 300 x g. The pelleted cells were resuspended in fresh medium and incubated in a 96-well plate for 5 days at 37°C to differentiate.

Adhesion molecules and flow cytometry

96-well polystyrene plates (BD Biosciences) were coated with 20 µg/mL of plasma fibronectin (SigmaAldrich) overnight at 4°C. The following day the coated wells were washed 3 x with PBS and blocked with 2% BSA in PBS. After blocking, the wells were washed 3x with PBS and then the PBMCs were plated with 2×10^5 cells per well in the presence or absence of 25 mM additional NaCl for 1 hour at 37°C. After the incubation, the medium was replaced with PBS/10 mM EDTA and the plate was left at 4°C for 15 minutes to detach the cells from the wells. The detached cells were then pelleted by centrifugation at 300 x g for 5 minutes and resuspended in 2% BSA in PBS. Following centrifugation, the cells were stained for CD11a (BioLegend, San Diego, CA), CD11b (BioLegend), CD11c (BioLegend), CD11b activation epitope (BioLegend), CD18 (BioLegend), CD18 activation epitope (Abd Serotec), CD31 (BioLegend), CD45 (BioLegend), CD49d (BioLegend) and, CD62L (BioLegend) as described previously [9].

Statistical analysis

Data was analyzed by ANOVA (with Dunnett's post test) or t-test when appropriate using Prism software (GraphPad software, San Diego, CA). Normality was tested using Shapiro-Wilk and D'Agostino-Pearson omnibus tests.

Author Contributions

Conceived and designed the experiments: RHG DP. Performed the experiments: NC DP. Analyzed the data: NC. Wrote the paper: NC.

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