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Murine T cell activation is regulated by surfen (*bis*-2-methyl-4-amino-quinolyl-6-carbamide)

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ABSTRACT

Surfen (*bis*-2-methyl-4-amino-quinolyl-6-carbamide) binds to glycosaminoglycans (GAGs) and has been shown to influence their function, and the function of proteoglycans (complexes of GAGs linked to a core protein). T cells synthesize, secrete and express GAGs and proteoglycans which are involved in several aspects of T cell function. However, there are as yet no studies on the effect of GAG-binding agents such as surfen on T cell function. In this study, surfen was found to influence murine T cell activation. Doses between 2.5 and 20 μM produced a graduated reduction in the proliferation of T cells activated with anti-CD3/CD28 antibody-coated T cell expander beads. Surfen (20 mg/kg) was also administered to mice treated with anti-CD3 antibody to activate T cells in vivo. Lymphocytes from surfen-treated mice also showed reduced proliferation and lymph node cell counts were reduced. Surfen reduced labeling with a cell viability marker (7-ADD) but to a much lower extent than its effect on proliferation. Surfen also reduced CD25 (the α-subunit of the interleukin (IL)-2 receptor) expression with no effect on CD69 expression in T cells treated in vivo but not in vitro. When receptor activation was bypassed by treating T cells in vitro with phorbyl myristate acetate (10 ng/ml) and ionomycin (100 ng/ml), surfen treatment either increased proliferation (10 μM) or had no effect (2.5, 5 and 20 μM). In vitro treatment of T cells with surfen had no effect on IL-2 or interferon-γ synthesis and did not alter proliferation of the IL-2 dependent cell line CTLL-2. The effect of surfen was antagonized dose-dependently by co-treatment with heparin sulfate. We conclude that surfen inhibits T cell proliferation in vitro and in vivo. When T cell receptor-driven activation is bypassed surfen had a neutral or stimulatory effect on T cell proliferation. The results imply that endogenous GAGs and proteoglycans play a complex role in promoting or inhibiting different aspects of T cell activation.

1. Introduction

Surfen (*bis*-2-methyl-4-amino-quinolyl-6-carbamide) was first described in 1938 as a component of depot insulin [1]; however, subsequent studies have revealed its efficacy in binding to glycosaminoglycans (GAGs). GAGs are composed of repeating disaccharide units (uronate:hexosamine) that vary in degree of acetylation and the pattern of *N* and *O*-sulphation; individual GAGs may contain 1–25,000 disaccharide units. All GAGs carry a net negative charge due to the presence of sulfate and carboxyl groups, and exist in soluble or cell-bound forms. When bound to the cell surface, GAGs form proteoglycans linked to a protein core by a linkage sequence (glucuronic acid–galactose–galactose–xylose) covalently bound to the protein core through serine residues. There is considerable interest in characterizing the biological function of GAGs, given their ubiquitous expression and ability to bind hundreds of proteins, including proteases, cytokines, adhesion molecules, cytokines and chemokines. The interaction is often charge-based since GAGs have a high affinity for cationic (basic) proteins; however, this is not the only mechanism because GAGs can also bind to anionic (acidic) proteins. The major classes of GAG are heparan sulfate, heparin, chondroitin sulfate, dermatan sulfate, keratin sulfate and hyaluronic acid [2].

Surfen contains four quinoline rings that contain positively charged amino or methyl groups. When characterized further, surfen was found to bind with greatest avidity to heparin, followed by dermatan sulfate, heparan sulfate and chondroitin sulfate [3]. There are now a handful of studies on the biological effects of surfen, many of which relate to its ability to block the interaction between GAGs and signaling proteins, including effects on growth

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factors (fibroblast growth factor and vascular endothelial growth factor) and fibrils associated with the binding of human immunodeficiency virus (HIV)-1 to target cells [3–5].

Although GAG synthesis, secretion and cell surface expression on T cells has been documented in several studies [6–15], the effects of a GAG binding inhibitor on T cells have yet to be reported. To our knowledge this study is the first to examine the effects of surfen on murine T cell activation. The results have implications for the function of GAGs in regulating T cell activation.

2. Materials and methods

2.1. Animals

Female C57/BL6 mice (6–8 weeks of age) were purchased from Charles River Canada (Lasalle, QC) and were used for all primary T cell experiments. All mice were housed in the Carleton Animal Care Facility at Dalhousie University. Animals were fed a standard diet of rodent chow and water ad libitum. All animal protocols were approved by the Dalhousie University Committee on Laboratory Animals and were in accordance with the Canadian Council on Animal Care guidelines.

2.2. Reagents and antibodies

Unless otherwise indicated, all chemicals including surfen were purchased from Sigma Aldrich Canada (Oakville, ON). A 100 mM stock solution of surfen was prepared in DMSO and stored at −80 °C. Phycoerythrin (PE)-conjugated anti-CD25 antibody (Ab), fluorescein isothiocyanate (FITC)-conjugated anti-CD69, PE-conjugated rat IgG1 and FITC-conjugated Armenian hamster IgG Abs were purchased from eBioscience Inc. (San Diego, CA).

2.3. T cell isolation

Mice were euthanized by cervical dislocation and axillary, brachial and inguinal lymph nodes pooled using two mice per sample. Lymph nodes were homogenized and $CD3^+$ T cells were isolated by negative selection using magnetic bead isolation columns from Miltenyi Biotech (Auburn, CA). Erythrocytes were removed by osmotic shock. Total cell numbers were assessed with trypan blue dye exclusion; cell viability was typically over 95%. For all experiments, T cells were cultured at 37 °C/5% CO_2/95% humidity in RPMI-1640 medium (Invitrogen, Burlington, ON) supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5 mM HEPES (all from Sigma–Aldrich).

2.4. CTLL-2 cells

Cytotoxic lymphoid line-2 (CTLL-2) cells were obtained from ATCC and were cultured in HEPES-free RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 30 U/ml of recombinant murine IL-2 (Pepro Tech Inc., Rocky Hill, NJ).

2.5. Tritiated-thymidine ([^{3}H]TdR) incorporation

To assess [^{3}H]TdR incorporation, T cells were cultured for 24, 48 or 72 h at 2.5×10^5 cells/well while CTLL-2 cells were cultured for 48 h at 1×10^4 cells/well in 96-well round-bottom plates with quadruplicate wells per treatment. Cells were stimulated with 5×10^4 anti-CD3/anti-CD28 Ab-coated T cell expander beads (Invitrogen) or combined phorbyl myristate acetate (PMA, 10 ng/ml) and ionomycin (100 ng/ml) with different doses of surfen or vehicle (0.02% DMSO) dissolved in serum free RPMI-1640 medium. For the last 6 h of incubation, cells were pulsed with 0.2 μCi of methyl [^{3}H]TdR (MP Biochemicals, Irvine, CA). Cells were harvested immediately onto fiberglass filter mats with a Titertek Cell Harvester (both from Skatron Instruments, Sterling, VA). [^{3}H]TdR incorporation into newly synthesized DNA was measured using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississuaga, ON).

2.6. Oregon Green proliferation assay

T cells were labeled with 2 μM Oregon Green 488 dye (Invitrogen) for 15 min at room temperature and then cultured in 96 well plates (1.5×10^5 cells/well) and stimulated for 72 h with 5×10^4 T cell expander beads. Cells were analyzed by flow cytometry using a FACSCaliber flow cytometer to calculate % proliferating cells.

2.7. Cytokine assays

T cells (1×10^5) were cultured in 96 well plates (200 μl/well) alone or in the presence of 5×10^4 T cell expander beads. Following 72 h incubation, culture supernatants were harvested and interleukin-2 (IL-2) and interferon-γ (IFN-γ) concentrations determined by ELISA (IL-2 kit from eBioscience, IFN-γ kit from BD Biosciences). All assays were performed in quadruplicate.

2.8. Cell viability assay

T cells were labeled with 7-ADD (0.25 μg in 5 μl) for 5 min then washed and analyzed by flow cytometry.

2.9. Staining for CD25 and CD69 expression

Cells were labeled on ice with fluorochrome-conjugated Abs or isotype matched fluorochrome-conjugated control Abs at a concentration of 0.5 μg in 50 μl FACS buffer (containing 0.2% NaN_3 and 1% BSA in PBS) for 45 min in the dark. Cells were then washed twice in FACS buffer, fixed in paraformaldehye solution and analyzed by flow cytometry.

2.10. In vivo administration of surfen

T cell activation was induced in vivo by intraperitoneal (ip) injection of each mouse with a 5 μg dose of anti-CD3 Ab (eBioscience). Mice were either treated with vehicle (0.1% DMSO) or with surfen (20 mg/kg, ip, both dissolved in serum free RPMI-1640 culture medium) by daily injection for 3 days prior to a single anti-CD3 Ab injection. They were killed 24 h later to harvest lymph nodes and obtain $CD3^+$ T cells using methods described above.

2.11. Statistical analysis

Comparisons between multiple data sets were performed with statistical software (GraphPad Prism) by one way analysis of variance with Bonferroni post-testing. Comparisons between two data sets were compared by Student's *t*-test, either paired or unpaired depending on experimental design; $p < 0.05$ was considered to be significant.

3. Results

3.1. Surfen reduces T cell proliferation in vitro and in vivo

$CD3^+$ murine T cells were stimulated with T cell expander beads, which resulted in a time-dependent increase in uptake of [^{3}H]TdR that peaked at 48 h (Fig. 1A). The co-addition of surfen during activation resulted in a dose-dependent reduction in [^{3}H]TdR incorporation. For wells stimulated for 48 h, incorporation was significantly

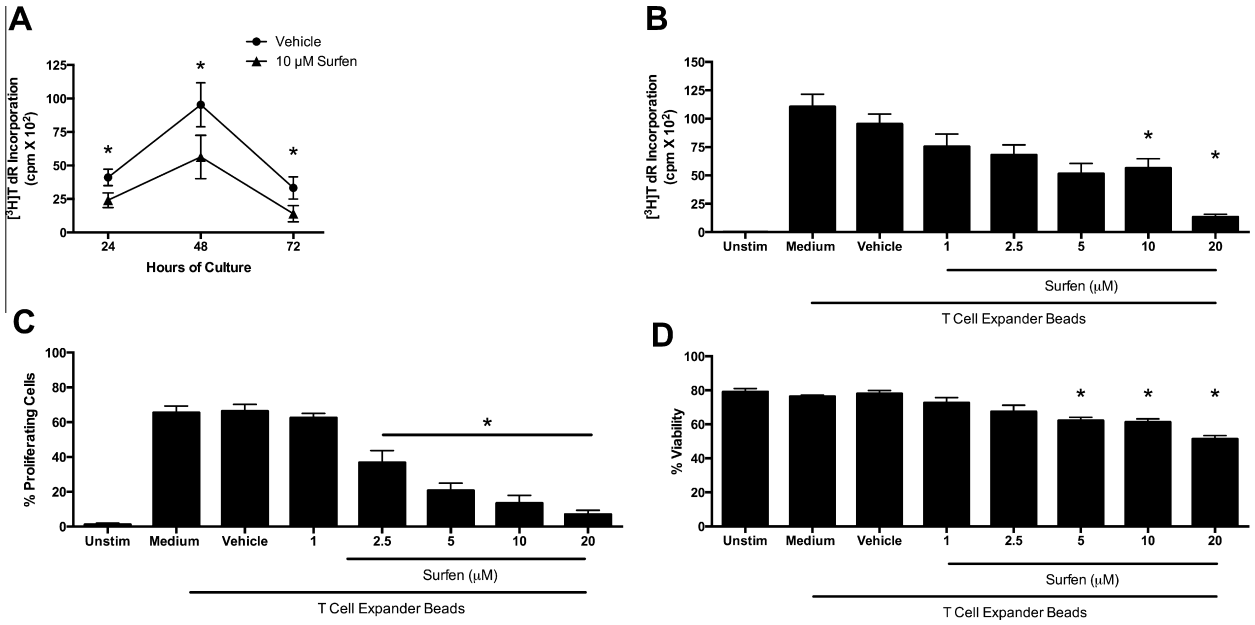

Fig. 1. Dose-dependent effects of surfen on activated murine lymphoid cells. (A) Graph showing time dependent effects of 10 μM surfen following microbead stimulation for 24, 48 and 72 h. (B) Bar chart showing tritiated thymidine ([^{3}H]TdR) incorporation for increasing doses of surfen after 48 h stimulation with T cell expander beads. (C) % of proliferating cells from Oregon Green incorporation for same doses. (D) Bar chart showing labeling with 7-ADD to assess toxicity for the same dose range. Data shown as mean ± SEM, $n = 4$ for all experiments. Significance is relative to vehicle control ($^*p < 0.05$).

reduced at 10 and 20 μM compared to vehicle (Fig. 1B). A similar pattern was noted for T cells stimulated for 24 h and 72 h (data not shown). Confirmation of the anti-proliferative effects of surfen was obtained by staining T cells with Oregon Green 488 dye, and analyzing the % of T cells undergoing proliferation (Fig. 1C). Once again, there was a dose-dependent reduction in proliferation compared to vehicle that was significant at 2.5, 5, 10 and 20 μM. To assess whether the reduced uptake of [^{3}H]TdR could be due to a toxic effect of surfen, T cells were stained with the viability marker 7-ADD (Fig. 1D). At a dose of 20 μM surfen induced the maximum reduction in cell numbers in which the mean is ca.33% lower than the vehicle group. By comparison, 20 μM surfen reduced the mean [^{3}H]TdR incorporation by ca.88% compared to vehicle, and mean % proliferation assessed with Oregon Green by ca.80% compared to vehicle. This indicates that the effects of surfen are largely not due to toxicity. To assess the in vivo efficacy of surfen, T cells were isolated from mice pre-treated with vehicle or surfen (20 mg/kg) for 3 days followed by anti-CD3 Ab injection and harvested 24 h later. As expected, treatment with anti-CD3 Ab induced significant uptake of [^{3}H]TdR, which was significantly reduced in the mice co-treated with surfen (Fig. 2A). In surfen treated mice, [^{3}H]TdR incorporation reverted to levels seen in untreated mice. This effect is also present when the data is expressed as a proliferation index, dividing results from treated animals by those from untreated mice (Fig. 2B). As additional confirmation, there was also a significant reduction in total cell counts for the lymph nodes removed from the surfen treated mice (Fig. 2C). These data show that surfen reduces T cell proliferation following activation in vitro and in vivo.

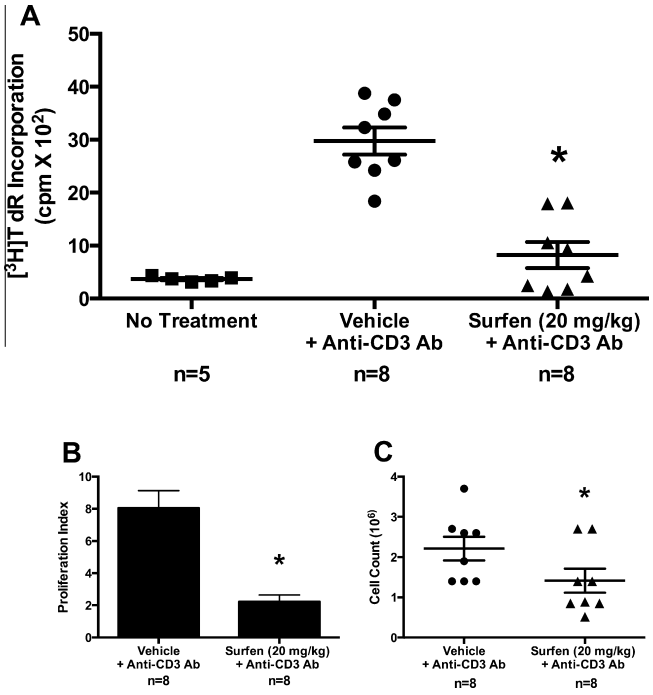

Fig. 2. Effect of surfen treatment (20 mg/kg) in mice treated with anti-CD3 Ab to induce T cell activation. (A) Scatter plot to show effect on [^{3}H]TdR incorporation. (B) Same data expressed as a proliferation index relative to untreated mice. (C) Effect on total lymph node cell counts. Data shown as mean ± SEM, n shown on the figures. Significance is relative to vehicle control ($^*p < 0.05$).

3.2. Surfen reduces CD25 expression in vivo but not in vitro

T cells were labeled to detect the proportion of cells that express CD25 and CD69. Mice treated with anti-CD3 Ab and surfen (20 mg/kg) showed reduced CD25 expression with no significant effect on CD69 compared to vehicle treated mice (Fig. 3A). In contrast, there was no observed reduction in CD25 or CD69 expression by isolated T cells stimulated in vitro with T cell expander beads in the absence or presence of 10 μM surfen (Fig. 3B). Additional information was obtained in CTLL-2 cells, whose growth is IL-2 dependent. Surfen between 1–20 μM had no significant effect on [^{3}H]TdR incorporation in these cells (data not shown). Surfen treatment (10 μM) also had no significant effect on release of IL-2 and IFN-γ into culture supernatants after T cell expander bead stimulation (data not shown).

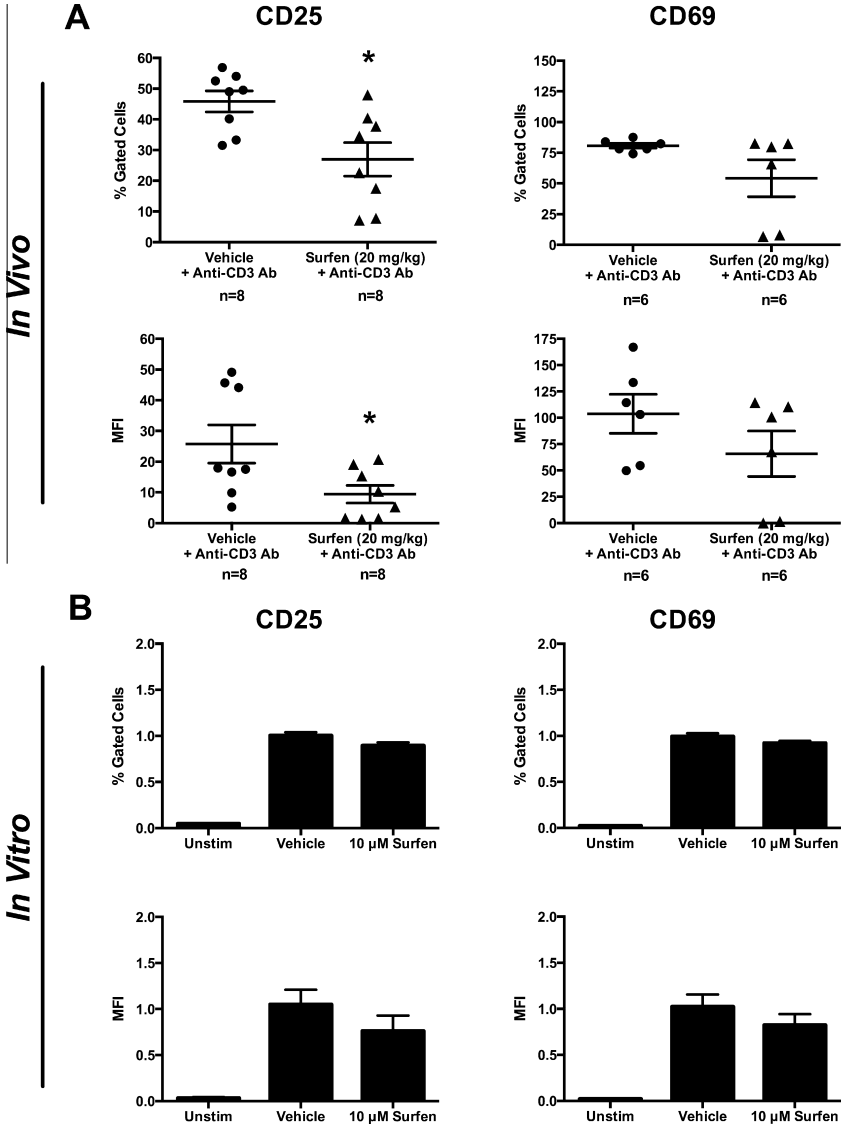

Fig. 3. Effect of 10 μM surfen on surface expression of CD25 and CD69. (A) Effect in T cells activated by anti-CD3 antibody in vivo. (B) Effect in T cells after stimulation in vitro with T cell expander beads for 48 h. Significance is relative to vehicle control (*$p < 0.05$).

3.3. The inhibitory effect of surfen is receptor-dependent and inhibited by heparin sulfate

Since surfen had effects on T cell receptor-driven proliferation, receptor activation was bypassed by stimulating T cells with PMA/ionomycin (Fig. 4A). Co-treatment with surfen either had no effect on proliferation (2.5, 5, 20 μM) or resulted in increased proliferation (10 μM). To explore the binding of surfen to GAGs and proteoglycans on T cells, surfen was co-applied with heparin sulfate at increasing doses which induced a dose-dependent reduction in the ability of 10 μM surfen to inhibit T cell proliferation after stimulation with T cell expander beads (Fig. 4B) or its ability to stimulate T cell proliferation after PMA/ionomycin treatment (Fig. 4C). Heparin sulfate alone had no effect on either response.

4. Discussion

4.1. Summary of results

When primary murine T cells were activated with T cell expander beads in the presence of surfen there was a dose-related inhibition of T cell proliferation (2.5–20 μM), as assessed from [^{3}H]TdR incorporation (Fig. 1A and B) and the fluorescence of cells stained with Oregon Green 488 dye (Fig. 1C). The impact of surfen was not simply due to cell toxicity (Fig. 1D). Surfen treatment in vivo had an identical effect on T cells that were activated by treating mice with anti-CD3 Ab. Surfen co-treatment (20 mg/kg) reduced T cell proliferation (Fig. 2A and B) and total cell counts in isolated lymph nodes (Fig. 2C). The molecular mechanisms include downregulation of CD25 (the α-subunit of the IL-2 receptor), resulting in a reduced ability to utilize growth-promoting IL-2 when studied in vivo (Fig. 3A) but not in vitro (Fig. 3B). Surfen had no effect on T cell release of the cytokines IL-2 and IFN-γ and did not reduce proliferation of IL-2-dependent CTLL-2 cells. When T cell receptor activation was bypassed by treating T cells with PMA/ionomycin, surfen induced a significant increase in proliferation (10 μM) or else had no effect (2.5, 5 and 20 μM; Fig. 4A). The effects of surfen on T cell receptor signaling appear to depend on binding to GAGs, since 10 μM surfen was blocked by co-application of the soluble GAG heparin sulfate in a dose-dependent manner (Fig. 4). These data establish an inhibitory effect of surfen on T cell activation in vitro and in vivo, but point to stimulatory effects when T cell receptor activation is bypassed.

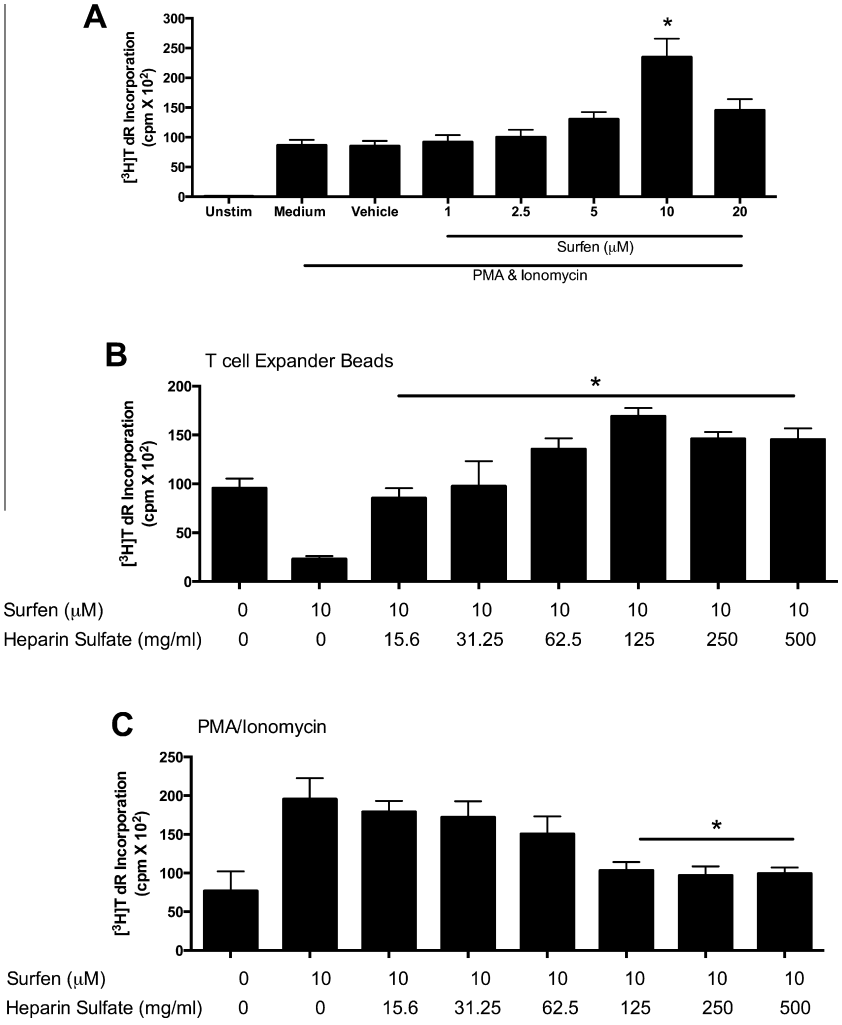

Fig. 4. Effects of surfen in T cells treated with PMA/ionomycin and inhibitory effect of heparin sulfate. (A) [^{3}H]TdR incorporation in T cells treated with PMA/ionomycin alone or in the presence of indicated doses of surfen. (B and C) 10 μM surfen was added alone or in combination with heparin sulfate at doses indicated following stimulation with T cell expander beads (B) or co-treatment with PMA/ionomycin (C). (A and C) Show means of replicate experiments ($n = 3–4$) while (B) is representative of 2 independent experiments. Significance ($^*p < 0.05$) is relative to vehicle (A) or to 10 μM surfen treatment alone (B and C).

4.2. Functional effects of surfen in other studies

Surfen antagonizes the effects of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), since both bind to heparan sulfate proteoglycans (HSPG) in order to potentiate interactions with receptor tyrosine kinases. Surfen reduced FGF binding to CHO cells and downstream phosphorylation of ERK in vascular endothelial cells (with an IC_{50} of about 5 μmol/L). Surfen also reduced tubule formation by vascular endothelial cells treated with FGF and VEGF, suggesting an ability to inhibit the formation of vascular tubes during angiogenesis [3]. Surfen inhibits the ability of VEGF to phosphorylate its receptor (VEGF-receptor 2) in primary mouse brain microvascular endothelial cells, and reduces the ability of VEGF to increase vascular permeability when injected into mouse skin [4]. Surfen also interferes with the ability of SEVI fibrils (semen-derived enhancer of viral infection) to bind to target cells to increase infectivity with human immunodeficiency (HIV) particles. In this example, surfen was shown to interact directly with the SEVI fibrils despite their net positive charge. This direct interaction bypassed the ability of surfen to prevent SEVI fibrils binding to cell surface HSPG [5].

4.3. The function of GAGs in T cells

GAGs have been implicated in several aspects of T cell function. T cells have been shown to synthesize HSPG and chondroitin sulfate proteoglycans (CSPG). Some of this is associated with the cell surface, while a component is released into the extracellular space. Although resting T cells synthesize proteoglycans, T cell activation results in a marked increase in mRNA expression, synthesis, release and surface expression [6–15]. The main categories of HSPG are the syndecans and glypicans, while serglycins form the major category of CSPG. The function of T cell surface proteoglycans has been explored in several contexts. Syndecan-2 and 4 have been shown to act as co-receptors for the entry of HIV-1 [16–19]. Other studies show involvement of T cell surface HSPG with the entry of human T cell leukemia virus (HTLV)-1 [12,20]. Several exogenous proteins have been found to bind either to T cells or to T cell lines. These include histones [21], cyclophilin B [22,23] and the chemokine RANTES [24]. Binding of a dendritic cell ligand (dendritic cell-associated heparan sulfate proteoglycan-dependent integrin ligand) to syndecan-4 on T cells reduces T cell proliferation and IL-2 production [25–29]. Binding of thrombospondin-1 to CD47 on Jurkat cells also has inhibitory effects [30]. Binding of cyclophilin B to T cell

HSPG induces integrin-mediated T cell adhesion to the extracellular matrix [22]. The binding of human group IIA phospholipase A_2 to HSPG on T cells in the early stages of apoptosis results in the generation of arachidonic acid [31]. Binding of RANTES to T cell HSPG inhibits entry of HIV-1 [24]. Cross-linking of syndecan-2 and -4 are on human T cells inhibits T cell proliferation and TNF-α production [12]. The function of secreted proteoglycans is less well described. However, secreted proteoglycans are complexed to perforin [32], granzyme A [33] and granzyme-B [34], all of which are involved in the function of cytotoxic T cells and natural killer cells. Exogenous CSPG has been found to worsen central nervous system inflammation during experimental autoimmune encephalomyelitis, while a disaccharide product of CSPG has the opposite effect [35]. In addition, T cell proliferation is inhibited when proteoglycan synthesis is inhibited by treatment with xyloside [7].

4.4. Implications of this work

The implication of these studies is that endogenous GAGs are involved in promoting T cell proliferation following T cell receptor driven-activation. The apparent binding of GAGs by surfen results in reduced expression of CD25 in vivo and a net inhibitory effect on T cell proliferation in vitro and in vivo (Figs. 1–3). However, surfen had the opposite effect on T cells activated with PMA/ionomycin, a treatment that induces T cell receptor-independent increases in intracellular calcium (Fig. 4). In this circumstance, the postulated binding of GAGs by 10 μM surfen resulted in an increase in T cell proliferation, suggesting that GAGs also play a role in regulating endogenous inhibitory mechanisms such as checkpoint inhibitors CTLA-4 and/or PD-1 [36]. The precise nature of the GAGs and proteoglycans involved are not known, although surfen has a preferential affinity for HSPG [3]. Surfen can also interact directly with cationic peptides involved in signaling, as shown for the study with SEVI fibrils [5], raising the possibility that its effects on T cell activation are independent of GAGs or proteoglycans. However, the inhibitory effect of heparin sulfate shows that when GAG binding sites on surfen are occupied by a competing moiety, surfen no longer exerts effects on T cell proliferation, whether inhibitory or stimulatory (Fig. 4). This suggests that GAG interactions are the primary mechanism for the actions of surfen on murine T cells. It is intriguing that in an earlier study [7] the treatment of T cells with xyloside, which inhibits proteoglycan synthesis, also inhibited T cell proliferation, suggesting that a stimulatory effect of GAGs and/or proteoglycans. Additional studies are warranted to explore the direct impact that endogenous GAGs and proteoglycans have on T cell function, but these are likely to be complex, involving both the promotion and inhibition of T cell proliferation depending on context.

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