

Chronic Wound Fluid Reduces Dermal Fibroblast Proliferation via a Mediated Signaling Pathway

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Abstract

Chronic wound fluid (CWF) from chronic venous leg ulcers has been shown to inhibit dermal fibroblast growth by interfering with cell-cycle progression from G1 to S phase. CWF was found to reduce the levels of hyperphosphorylated retinoblastoma tumor-suppressor gene (Rb) and cyclin D1, both of which are required for the cell cycle to enter the S phase. To better understand the effects of CWF, researchers looked into a Ras-mediated signalling pathway involving the mitogen-activated protein kinase kinase (MEK), which is known to modulate the expression of these cell-cycle-regulatory proteins [1-15]. The growth suppressive effects of CWF on hyperphosphorylated Rb (ppRb) and cyclin D1 were abolished by transient transfection of dermal fibroblasts with constitutively active Ras. In comparison, the effects of CWF on these cell-cycle-regulatory proteins were mimicked by a MEK inhibitor, PD 98059. Concurrent administration of PD 98059 and CWF resulted in additive effects. These findings suggest that CWF inhibits dermal fibroblast growth, at least in part, by decreasing the level of active Ras, which results in lower levels of ppRb and cyclin D1. As a result, a Ras-dependent signalling pathway may mediate the growth inhibitory effect of CWF, and restoring Ras activity may overcome this effect. Wound fluid is thought to play an important role in wound healing. Acute wound fluid has been shown to stimulate fibroblast and endothelial cell growth, induce chemotaxis and increase extracellular matrix production. In contrast, chronic wound fluid (CWF) has been shown to inhibit cellular proliferation contributing to the poor healing of chronic ulcers. CWF inhibits the proliferation of newborn dermal fibroblasts (NbFb) and DNA synthesis in human neonatal fibroblasts, and it halts the cell cycle in the G1 phase (Phillips et al, 1998). Fibroblast proliferation is critical to wound healing, and any disruption can significantly alter proper wound healing.

The tightly regulated eukaryotic cell cycle can be broadly divided into two phases: S (DNA synthesis) and M (mitosis), with a gap phase before S called G1 and a gap phase after M called G2. The product of the retinoblastoma tumor-suppressor gene (Rb), which modulates cell-cycle progression by sequestering transcription factors that regulate transcription of genes. The transcription factors are released, allowing the cell to progress from G1 to S phase (Weinberg, 1995). The cyclin D1/CDK4 cyclin-dependent kinase complex is known to phosphorylate Rb and studies show that the cyclin D1/CDK4 complex is required for G1 progression in mammalian cells (Baldin et al, 1993; Sherr, 1993). The cyclin D1/CDK4 complex is made up of a catalytic subunit called CDK4 and a regulatory cyclin called cyclin D1. Phosphorylation and association with a regulatory cyclin subunit are required for the complex to be activated. According to a recent report, it has been proposed that CWF inhibits dermal fibroblast growth by decreasing the levels of cell-cycle-regulatory proteins such as ppRb and cyclin D1.

Ras, a guanine nucleotide-binding protein with a molecular weight of 21kD, is a key regulator of cell growth in all eukaryotic cells (Lowy and Willumsen, 1993). Ras receives signals from a wide range of extracellular stimuli, and Ras mediates its effects by activating a cascade of protein kinases (acts as a molecular switch at the plasma membrane's inner leaflet, and its activity is regulated by a guanosine triphosphate (GTP)-bound protein (MAP) kinase pathway. Active Ras binds inactive Raf and translocates it to the plasma membrane where the Raf is activated. (Many studies have established that cyclin D1 expression is induced by Ras through a Raf/MEK/MAP kinase-dependent pathway. The ability of oncogenic Ras to shorten the G1 phase can be attributed to increased induction of cyclin D1. Furthermore, expression of dominant-negative Ras into cycling cells causes a decline in cyclin D1, accumulation of hypophosphorylated Rb and subsequent growth arrest in G1, which can be overcome with induction of the pathway. Mitogen-activated protein (MAP) kinase. Active Ras binds inactive Raf and transports it to the plasma membrane, where it activates Raf. Many studies have shown that Ras induces cyclin D1 expression via a Raf/MEK/MAP kinase pathway and that the ability of oncogenic Ras to shorten the G1 phase can be attributed to increased induction of cyclin D1.)

Introduction

To compare the effects of CWF and acute wound fluid on NbFb proliferation, 1500 cells per 35 mm culture dish were plated in paired cultures. Cells were then treated with acute wound fluid, CWF, or bovine serum albumin (BSA) at a protein concentration of 250 g per plate, and at each refeeding, fresh CWF, acute wound fluid, or BSA was added. The total cell number per culture dish was determined using a Coulter particle Counter on days 7 and 10 after plating. Acute wound fluid stimulated the growth of NbFb within 7 days of treatment, and by day 10, cells treated with acute wound fluid had a significantly higher number of cells per dish than BSA-treated cells.

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Subjective Heading

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Discussion

TdT-mediated dNTP Nick end labelling (TUNEL) assay was used to confirm that CWF does not cause apoptosis in NbFb. Paired cultures of NbFb were treated with either BSA or CWF at a concentration of 250 g protein per mL media. After 24 hours of treatment, cells were processed using FACS to determine the level of fluorescence, which indicates apoptosis. When the FACS analysis profile was superimposed on BSA- and CWF-treated cells, the number of cells and fluorescence intensity were identical between the two samples. These findings support the notion that CWF does not cause apoptosis in NbFb cells.

Because Ras is important in mediating mitogenic activation of the cell-cycle machinery, the effects of CWF on Ras protein levels were investigated. Quiescent NbFb were stimulated to proliferate in the presence of either CWF or BSA with 10% CS. Cells were harvested 16 and 20 hours after serum stimulation for immunoblot analysis with a specific monoclonal antibody against Ras. At all time points studied, serum stimulation did not increase the level of Ras protein above that of quiescent cells. Surprisingly, CWF had no effect on Ras protein levels. To determine whether CWF affects Ras activity, an assay based on the known specificity of the interaction between Ras-GTP and the Ras-binding domain (RBD) of Raf-1 was used to detect activated Ras. Quiescent NbFb were stimulated to proliferate in the presence of either CWF or BSA with 10% CS. Cells were harvested 6 and 16 hours after stimulation, and lysates were incubated with a glutathione-S-transferase-RBD fusion protein (GST-RBD) immobilised on glutathione-sepharose to precipitate the active GTP-bound form of Ras. Immunoblotting was used to detect precipitated Ras. As previously reported the level of active Ras protein in quiescent NbFb was below

detectable increase the level of active Ras at 6 and 16 hours by fourfold. The level of active Ras in CWF-treated cells remained low at both time points, similar to that in quiescent cells (Figures 4a and b), and significantly lower than that in BSA-treated cells. These findings imply that CWF may exert its effects by inhibiting Ras activity.

To confirm that CWF effects are mediated by a Ras-dependent signalling pathway, the constitutively active myc-tagged (9E10) Ras38V mutant was transiently transfected into NbFb. A group of NbFb were first synchronised into quiescence, then stimulated to proliferate for 24 hours before transfection with 10% CS. The cells were then treated with BSA or CWF (250 g) 40 hours after transfection. As previously reported, CWF significantly reduced the level of cyclin D1 in non-transfected NbFb when compared to BSA. The constitutively active Ras38V mutant, on the other hand, abolished the effect of CWF on cyclin D1 and was comparable to BSA-treated controls ($p > 0.1$, Figure 5b). Serum stimulation of cells expressing constitutively active Ras38V did not result in a higher level of cyclin D1 than cells expressing only endogenous Ras.

Rb and ppRb levels were measured in parallel using the same cell lysates used for cyclin D1 immunoblot analysis. Rb shifted to ppRb in non-transfected cells in response to serum stimulation, as expected, and this shift was inhibited by CWF. Because cyclin D1 is regulated by a Ras-dependent signalling pathway involving MAP kinase, PD 98059 was used to investigate the potential role of this pathway in mediating CWF effects. PD 98059 is a non-competitive inhibitor of MEK activity that can prevent MEK from phosphorylating MAP kinase without affecting the activity of already phosphorylated MAP kinase. Quiescent NbFb were pre-treated for 30 minutes with 10 M PD 98059 before adding 10 percent CS with either CWF or BSA (250 g per mL) as a control. Cells were harvested for immunoblot analysis at 16 and 24 hours after stimulation.

Serum stimulation increased the level of cyclin D1, as expected, and CWF inhibited this increase. Similarly, PD 98059 suppressed cyclin D1 levels at 16 and 24 hours indicating that MEK inhibition alone could significantly block serum-induced increases in cyclin D1 levels ($p < 0.05$ to $p < 0.01$) mimicking the effect of CWF. Furthermore, treatment with both CWF and PD 98059 had an additive effect, significantly decreasing the level of cyclin even to levels lower than in quiescent cells. At higher concentrations of PD 98059, the serum-induced increase in cyclin D1 was completely blocked.

CWF has been shown to specifically inhibit proliferation of dermal fibroblasts and endothelial cells, thus slowing healing whereas acute wound fluid has been shown to stimulate proliferation of fibroblasts and endothelial cells. Our findings in paired cultures confirmed the opposing effects of CWF and acute wound fluid on fibroblast proliferation. These findings highlight the critical roles that the wound microenvironment, such as wound fluid, may play during the healing process. However, the molecular mechanisms by which CWF inhibits cell proliferation in various cell types are not well understood. We investigated the role of a Ras-dependent signalling pathway in this study.

Conclusion

CWF significantly reduced the level of active Ras in serum stimulated NbFb, according to our findings. It is well established that activation of the Ras-dependent Raf/MEK pathway is responsible for cyclin D1 upregulation and the ability to induce Rb phosphorylation. As a result, a Ras-dependent pathway may be the primary effector pathway mediating CWF's effects on ppRb and cyclin D1 levels.

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Conflict of Interest

The authors declare that they are no conflict of interest.

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