Chronic Wound Fluid Suppresses Proliferation of Dermal Fibroblasts Through a Ras-Mediated Signaling Pathway

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Wound fluid collected from chronic venous leg ulcers (chronic wound fluid (CWF)) has been shown to inhibit the growth of dermal fibroblasts by interfering with cell-cycle progression from G1 into S phase. Specifically, CWF was shown to downregulate the levels of hyperphosphorylated retinoblastoma tumor-suppressor gene (Rb) and cyclin D1, known to be critical for entering the S phase of the cell cycle. To further elucidate the effects of CWF, a Ras-mediated signaling pathway involving the mitogen-activated protein kinase kinase (MEK), known to modulate the expression of these cell-cycle-regulatory proteins, was examined. Transient transfection of dermal fibroblasts with constitutively active Ras abrogated the growth suppressive effects of CWF on hyperphosphorylated Rb (ppRb) and cyclin D1. In contrast, an MEK inhibitor PD 98059 mimicked the effects of CWF on these cell-cycle-regulatory proteins. Concurrent treatment with PD 98059 and CWF produced additive effects. Taken together, these results suggest that CWF inhibits the growth of dermal fibroblasts at least in part by decreasing the level of active Ras, resulting in decreased levels of ppRb and cyclin D1. Therefore, a Ras-dependent signaling pathway may mediate the growth inhibitory effect of CWF, and reconstitution of Ras activity may overcome this growth inhibitory effect.

Key words: proliferation/Ras/venous leg ulcers/wound healing
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Wound fluid is thought to play a critical role in the wound-healing process (Falanga, 1992; Schultz and Mast, 1998). Acute wound fluid has been shown to stimulate the growth of fibroblasts and endothelial cells (Greenburg and Hunt, 1978; Jalkanen *et al*, 1983; Katz *et al*, 1991), induce chemotaxis (Banda *et al*, 1982), and increase production of extracellular matrix (Jalkanen *et al*, 1983; Katz *et al*, 1991). In contrast, wound fluid from chronic wounds (chronic wound fluid (CWF)), has been shown to inhibit cellular proliferation (Bucalo *et al*, 1993), contributing to the impaired healing of chronic ulcers (Palolahti *et al*, 1993; Phillips *et al*, 1998). CWF inhibits the proliferation of newborn dermal fibroblasts (NbFb) (Bucalo *et al*, 1993; Mendez *et al*, 1999), inhibits DNA synthesis in human neonatal fibroblasts, and arrests cells in the G1 phase of the cell cycle (Phillips *et al*, 1998). Fibroblast proliferation is critical to the healing process (Clark, 1989; Gross *et al*, 1995; McClain *et al*, 1996), and any disruption of fibroblast proliferation can dramatically alter proper wound healing.

The tightly regulated eukaryotic cell cycle can be broadly divided into an S (DNA synthesis) phase and an M (mitosis) phase, with a gap phase before S phase termed G1, and a gap after M phase termed G2. One of the most important regulators of G1 phase progression is the product of the retinoblastoma tumor-suppressor gene (Rb), which modulates cell-cycle progression by sequestering transcription factors that regulate transcription of genes required for entry into S phase, such as E2F and histon deacetylase (Nevins, 1992; Hinds and Weinberg, 1994; Weinberg, 1995). When Rb is hyperphosphorylated (ppRb), it releases the transcription factors, allowing passage of the cell through G1 into the S phase (Weinberg, 1995). Rb is known to be phosphorylated by the cyclin D1/CDK4 cyclin-dependent kinase complex (Kato *et al*, 1993; Sherr, 1993; Matsushime *et al*, 1994), and studies show that the cyclin D1/CDK4 complex is critical for G1 progression in mammalian cells (Baldin *et al*, 1993; Sherr, 1993). The cyclin D1/CDK4 complex is composed of a catalytic subunit, CDK4, and a regulatory cyclin subunit, cyclin D1 (Nasmyth, 1993). Activation of the complex requires phosphorylation and association with a regulatory cyclin subunit (Nasmyth, 1993). A recent report has suggested that CWF suppresses the growth of dermal fibroblasts by decreasing the levels of cell-cycle-regulatory proteins, including ppRb and cyclin D1 (Seah *et al*, 2001).

Ras, a 21kD guanine nucleotide-binding protein, is a key regulator of cell growth in all eukaryotic cells (Lowy and Willumsen, 1993). Signaling pathways from a diverse array of extracellular stimuli converge on Ras, and Ras mediates its effects through the activation of a cascade of protein kinases (Campbell *et al*, 1998; Vojtek and Der, 1998). Ras

Abbreviations: BSA, bovine serum albumin; CWF, chronic wound fluid; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; GTP, guanosine triphosphate; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; NbFb, newborn dermal fibroblasts; ppRb, hyperphosphorylated Rb; Rb, retinoblastoma tumor-suppressor gene; RBD, Ras-binding domain; TNF-α, tumor necrosis factor; TUNEL, TdT-mediated dNTP Nick end labeling

functions as a molecular switch positioned at the inner leaflet of the plasma membrane, and its activity is controlled by a regulated guanosine diphosphate (GDP)/guanosine triphosphate (GTP) cycle (Wittinghofer and Pai, 1991; Ma and Karplus, 1997). The majority of Ras in quiescent cells is in the inactive GDP-bound state, and stimulation of cells with mitogens or serum increases the abundance of the active GTP-bound state (Satoh *et al*, 1990a, b). The first identified, and perhaps the best-characterized, downstream effector of Ras is the protein kinase Raf that phosphorylates a mitogen-activated protein kinase kinase (MEK) and propagates the mitogen-activated protein (MAP) kinase pathway (Macdonald *et al*, 1993). Active Ras binds inactive Raf and translocates it to the plasma membrane where the Raf is activated (Stokoe *et al*, 1994). Many studies have established that cyclin D1 expression is induced by Ras (Filmus *et al*, 1994; Liu *et al*, 1995; Arber *et al*, 1996; Winston *et al*, 1996; Aktas *et al*, 1997; Fan and Bertino, 1997; Kawada *et al*, 1997) through a Raf/MEK/MAP kinase-dependent pathway (Lavoie *et al*, 1996; Cheng *et al*, 1998) and the ability of oncogenic Ras to shorten the G1 phase can be attributed to increased induction of cyclin D1 (Liu *et al*, 1995; Winston *et al*, 1996). 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 cyclin D1 expression (Peeper *et al*, 1997). These findings provide strong evidence that Ras-dependent signaling plays a critical role in Rb phosphorylation, likely by modulating the levels of cyclin D1. Although the ability to induce Rb phosphorylation through activation of cyclin D-dependent kinases does not account for all Ras effects on the cell cycle, Ras control of cyclin D1 expression does appear to be a major part of the link between Ras and the cell-cycle machinery (Aktas *et al*, 1997).

CWF has been shown to inhibit the proliferation of dermal fibroblasts by arresting cells in the G0/G1 phase of the cell cycle (Phillips *et al*, 1998). In this paper, the modulation of ppRb and cyclin D1 by CWF through a Ras-dependent pathway was investigated.

Results

Acute wound fluid stimulated proliferation of NbFb To compare the effects of CWF and acute wound fluid on the proliferation of NbFb, paired cultures of NbFb were plated at 1500 cells per 35 mm culture dish. Cells were then treated with acute wound fluid, CWF or bovine serum albumin (BSA) at 250 μg protein per plate, and at each refeeding fresh CWF, acute wound fluid or BSA was supplemented. At days 3, 5, 7, and 10 after plating, total cell number per culture dish was determined using Coulter particle Counter. Within 7 d of treatment, acute wound fluid began to stimulate the growth of NbFb, and by day 10, cells treated with acute wound fluid displayed a significantly higher number of cells per dish when compared with BSA-treated cells (Fig 1). This result is consistent with previous reports that acute wound fluid stimulated the growth of fibroblast and endothelial cells (Jalkanen *et al*, 1983; Katz *et al*, 1991; Greenburg and Hunt, 1978). In contrast, CWF suppressed the growth of NbFb (Fig 1) as previously reported (Bucalo *et al*, 1993; Phillips *et al*, 1998; Mendez *et al*, 1999).

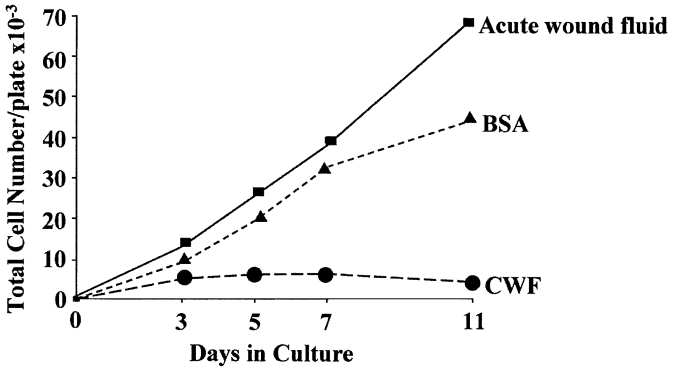

Figure 1
Effects of acute wound fluid and chronic wound fluid (CWF) on the proliferation of newborn dermal fibroblasts (NbFb). Paired cultures of NbFb were plated at 1500 cells per 35 mm dish. Cells were then treated with Bovine serum albumin, acute wound fluid or CWF at 250 μg per dish. Total cell number per plate was determined using Coulter Particle Counter on the indicated days. A representative result from three independent experiments is shown. Acute wound fluid among the patients was not pooled.

CWF did not induce apoptosis To investigate whether the CWF-induced suppression of NbFb growth may be, in part, because of apoptosis, fluorescence-activated cell sorter (FACS) analysis was performed on BSA- and CWF-treated NbFb. NbFb serum starved (0.1% calf serum (CS)) for 24 h and NbFb incubated with 0.1 μM staurosporine, known to induce apoptosis (Tepper *et al*, 2000), for 6 h, were used as controls. Apoptotic cells were quantified by FACS analysis, using propidium iodide (PI) staining, as previously described (Krishan, 1975; Hotz *et al*, 1994).

In staurosporine-treated NbFb, a large percentage of cells were apoptotic cells as the FACS profile showed a significant sub-G1 (hypodiploid) peak, characteristic of apoptotic cells (Fig 2*Aa*). As expected, in quiescent cells more than 75% of cells were in the G1 phase with only 11.2% of cells in S phase (Fig 2*Ab*). In contrast, proliferating NbFb, stimulated with 10% CS in the presence of BSA, had a higher percentage of cells in S phase (23.8%) (Fig 2*Ac*). CWF treatment caused an accumulation of cells mostly in G1 (65.5%), and to a much lesser extent, in G2 (27%) (Fig 2*Ad*), as previously reported. No significant accumulation of cells in sub-G1 was, however, observed for CWF-treated cells, suggesting that CWF does not induce apoptosis.

To further confirm that CWF does not cause apoptosis in NbFb, TdT-mediated dNTP Nick end labeling (TUNEL) assay was performed. Paired cultures of NbFb plated at 1×10^{-6} cells per 100 mm were treated with either BSA or CWF at 250 μg protein per mL media. After 24 h of treatment, cells were then processed to assess the level of fluorescence, indicative of apoptosis, using FACS. When the profile of FACS analysis was overlayed between BSA- and CWF-treated cells, the number of cells and the intensity of fluorescence was identical between two samples (Fig 2*B*). These results further confirm that CWF does not induce apoptosis in NbFb cells.

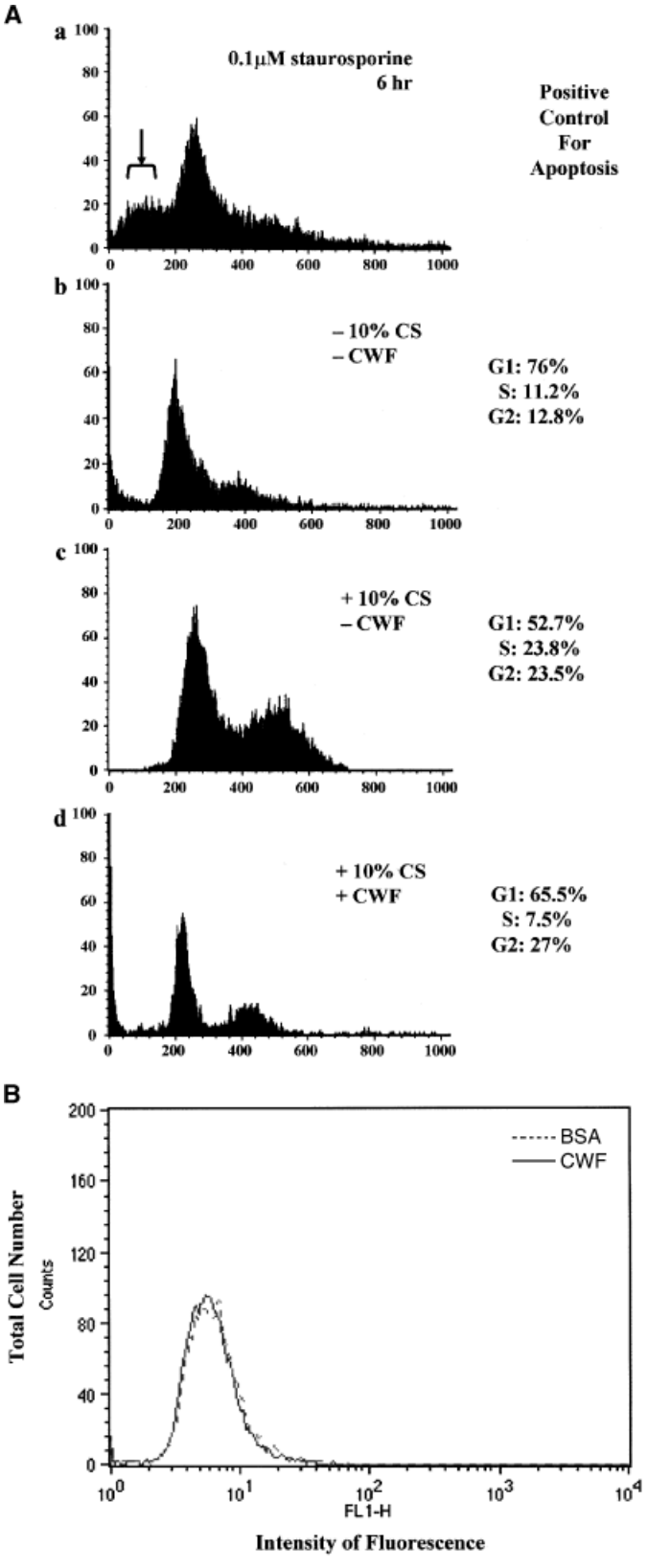

Figure 2
Fluorescence-activated cell sorter (FACS) analysis profiles for proliferating and apoptotic cells. (*A*) Treatment with 0.1 μM staurosporine for 6 h induced apoptosis in newborn dermal fibroblasts (NbFb), and arrows indicate the sub-G1 peaks characteristic of apoptotic cells (*a*). FACS profiles for serum-starved NbFb (*b*), proliferating NbFb (*c*), and chronic wound fluid (CWF)-treated NbFb (*d*) are shown for comparison. (*B*) TUNEL assay was performed on cells treated with BSA or CWF on subconfluent culture of NbFb.

Effect of CWF on the level of Ras protein Since Ras plays a central role in mediating mitogenic activation of the cell-cycle machinery, the effects of CWF on the level of Ras protein were examined. Quiescent NbFb were stimulated to proliferate with 10% CS in the presence of either CWF or BSA. Sixteen and 20 h after serum stimulation, cells were harvested for immunoblot analysis using specific monoclonal antibody against Ras. Serum stimulation did not increase the level of Ras protein from that of quiescent cells at all time points examined. Interestingly, CWF had no effect on the level of Ras protein (Fig 3).

Effect of CWF on Ras activity To examine 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 (Warne *et al*, 1993; Chuang *et al*, 1994; Hallberg *et al*, 1994; Pumiglia *et al*, 1995) was used to detect activated Ras as previously described (Taylor and Shalloway, 1996). Quiescent NbFb were stimulated to proliferate with 10% CS in the presence of either CWF or BSA. At 6 and 16 h after stimulation, cells were harvested and lysates incubated with a glutathione-S-transferase–RBD fusion protein (GST–RBD) immobilized on glutathione–sepharose to precipitate the active GTP-bound form of Ras. Precipitated Ras was detected by immunoblotting. The level of active Ras protein in quiescent NbFb was below detectable levels, as previously reported (Satoh *et al*, 1990a, b). Serum stimulation increased the level of active Ras at 6 and 16 h by 4-fold (Fig 4*A*). In CWF-treated cells, the level of active Ras remained low at both time points, similar to that in quiescent cells (Figs 4*A* and *B*), and significantly lower than that in BSA-treated cells ($p < 0.05$) (Fig 4*B*). These results suggest that CWF may be exerting its effects by inhibiting Ras activity.

Constitutively active Ras abrogates effects of CWF To further confirm that a Ras-dependent signaling pathway mediates the effects of CWF, the constitutively active myc-tagged (9E10) Ras38V mutant was transiently transfected into NbFb. A set of NbFb were first synchronized into quiescence, then stimulated to proliferate with 10% CS for 24 h before transfection. 40 h after transfection the cells were then treated with BSA or CWF (250 μg per mL) for 16 h. Immunoblot analyses indicate that the expression of transfected Ras38V, as indicated by myc expression, was

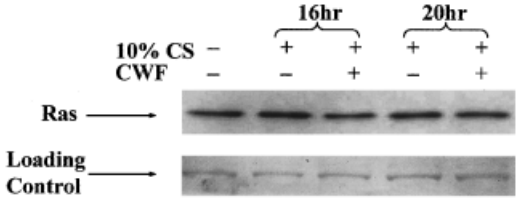

Figure 3
Chronic wound fluid (CWF) does not affect the level of Ras protein. Paired cultures of newborn dermal fibroblasts were treated with either bovine serum albumin or CWF in the presence of 10% calf serum (CS), and cell lysates were subjected to immunoblot analysis. A representative blot of five independent experiments is shown. As a loading control, membrane was stained with Coomassie blue staining solution after immunoblot analysis was completed.

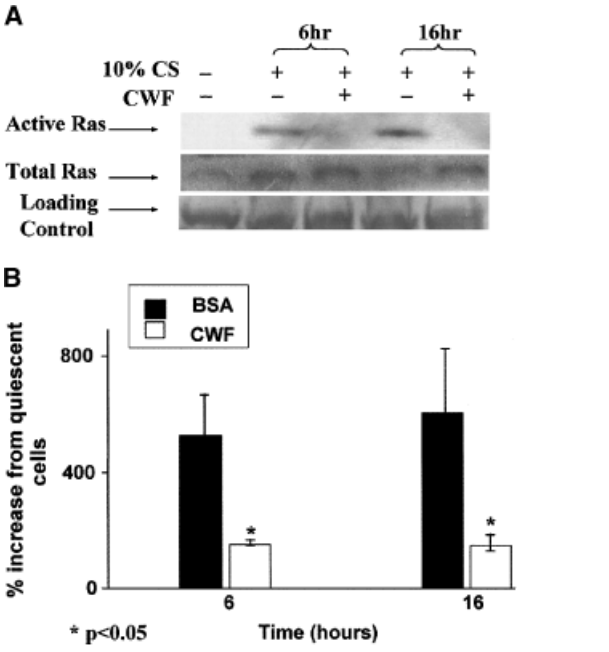

Figure 4
Chronic wound fluid (CWF) suppressed the serum-induced level of active Ras. (*A*) Paired cultures of newborn dermal fibroblasts were treated with either bovine serum albumin (BSA) or CWF in the presence of 10% calf serum (CS). Active Ras precipitated with Raf-1-Ras-binding domain reagent was detected by immunoblot analysis. A representative blot from three independent experiments is shown. From the same lysate, the level of total Ras was determined. As a loading control, membrane was stained with Coomassie blue staining solution after the immunoblot analysis was completed. (*B*) Immunoblot results from three independent experiments were subjected to densitometric analysis and values were averaged and statistical analysis was performed for each time point to compare active Ras levels in BSA- and CWF-treated cells. Two-way ANOVA for repeated measures, for both time and treatment variables, showed CWF with significantly lowered active Ras levels ($\alpha = 0.05$, $p < 0.001$).

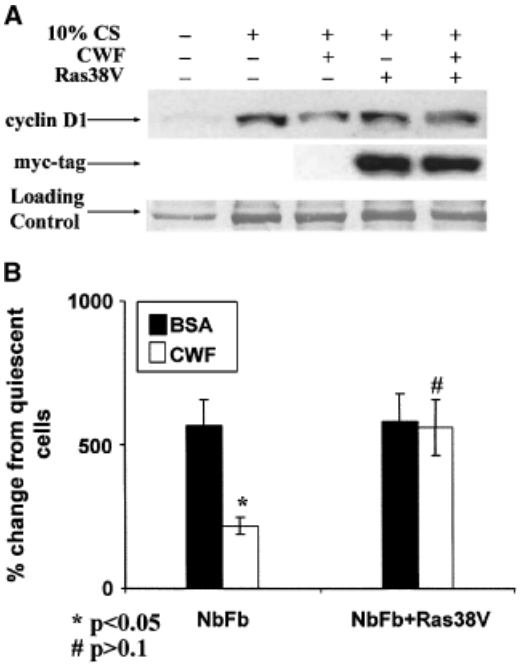

Figure 5
Effect of chronic wound fluid (CWF) on the level of cyclin D1 in newborn dermal fibroblasts (NbFb) expressing constitutively active Ras38V. (*A*) Non-transfected and Ras38V-transfected NbFb were treated with either bovine serum albumin (BSA) or CWF 40 h after transfection, and harvested for immunoblot analysis 16 h after treatment. A representative blot of three independent experiments is shown. As a loading control, membranes were stained with Coomassie blue staining solution after exposing the membrane to the X-OMAT film. (*B*) Immunoblot results from three independent experiments were subjected to densitometric analysis and values were averaged and statistical analysis was performed for each time point to compare cyclin D1 levels in BSA- and CWF-treated cells. The two-tailed, paired Student's *t* test showed a statistically significant lower level of cyclin D1 in CWF-treated cells compared with BSA-treated cells ($p < 0.05$), but in cells expressing constitutively active Ras38V, the effect of CWF on the level of cyclin D1 was abrogated ($p > 0.1$).

comparable between BSA- and CWF-treated cells (Fig 5*A*). The levels of cyclin D1 and ppRb, which reflect the downstream effects of Ras activation, were examined.

In non-transfected NbFb, CWF significantly reduced the level of cyclin D1 as compared with BSA ($p < 0.05$, Fig 5*B*), as previously reported. Expression of the constitutively active Ras38V mutant, however, abrogated the effect of CWF on cyclin D1 (Fig 5*A*), and was comparable with that of BSA-treated controls ($p > 0.1$, Fig 5*B*). Serum stimulation of cells expressing constitutively active Ras38V did not cause a further increase in cyclin D1 compared with cells expressing only endogenous Ras (Figs 5*A* and *B*).

The levels of Rb and ppRb were analyzed in parallel, using the same cell lysates harvested for cyclin D1 immunoblot analysis. In non-transfected cells, Rb shifted to ppRb in response to serum stimulation, as expected, and this shift was inhibited by CWF (Fig 6*A*). Expression of constitutively active Ras38V blocked the effect of CWF on ppRb (Fig 6*A*). CWF significantly inhibited the induction of ppRb as compared with BSA control ($p < 0.05$), but in Ras38V expressing cells, CWF did not cause any significant inhibition of the level of ppRb ($p > 0.1$) (Fig 6*B*).

MEK Inhibition Mimics CWF Effects Since cyclin D1 is regulated by a Ras-dependent signaling pathway involving MAP kinase, the possible role of this pathway in mediating CWF effects was examined, using PD 98059. PD 98059 is a specific, non-competitive inhibitor of MEK activity, able to block phosphorylation of MAP kinase by MEK without affecting the activity of already phosphorylated MAP kinase. Quiescent NbFb were pre-treated with 10 μM PD 98059 for 30 min prior to the addition of 10% CS with either CWF or BSA (250 μg per mL) as control. Cells were then harvested at 16 and 24 h after stimulation for immunoblot analysis.

As expected, serum stimulation caused an increase in the level of cyclin D1 and CWF inhibited this increase (Fig 7*A*, *lanes 1–3* and *6*). Similarly, PD 98059 suppressed the level of cyclin D1 at 16 and 24 h (Fig 7*A*, *lanes 3*, *4*, *6*, and *7*), indicating that MEK inhibition alone could significantly block serum-induced increase in the level of cyclin D1 ($p < 0.05$ to $p < 0.01$) (Fig 7*B*), mimicking the effect of CWF ($p < 0.05$ to $p < 0.01$) (Fig 7*B*). Moreover, treatment with both CWF and PD 98059 produced an additive effect, significantly down-regulating the level of cyclin D1 ($p < 0.01$) (Fig 7*B*), even below the level in quiescent cells (Figs 7*A* and *B*). A higher concentration of PD 98059 completely blocked the serum-induced increase in cyclin D1 at both 16 and 24 h (data not shown).

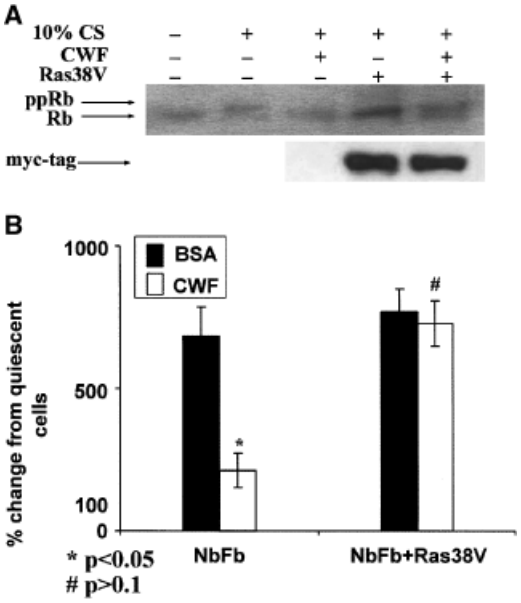

Figure 6
Effect of chronic wound fluid (CWF) on the level of hyperphosphorylated Rb (ppRb) in newborn dermal fibroblasts (NbFb) expressing constitutively active Ras38V. (*A*) Non-transfected and Ras38V-transfected NbFb were treated with either bovine serum albumin (BSA) or CWF 40 h after transfection, and harvested for immunoblot analysis 16 h after treatment. A representative blot of three independent experiments is shown. (*B*) Immunoblot results from three independent experiments were subjected to densitometric analysis and values were averaged and statistical analysis was performed for each time point to compare ppRb levels in BSA- and CWF-treated cells. The two-tailed, paired Student's *t* test showed CWF significantly inhibited the induction of ppRb as compared with BSA control ($p < 0.05$), but in Ras38V expressing cells, CWF did not cause any significant inhibition of the level of ppRb ($p > 0.1$).

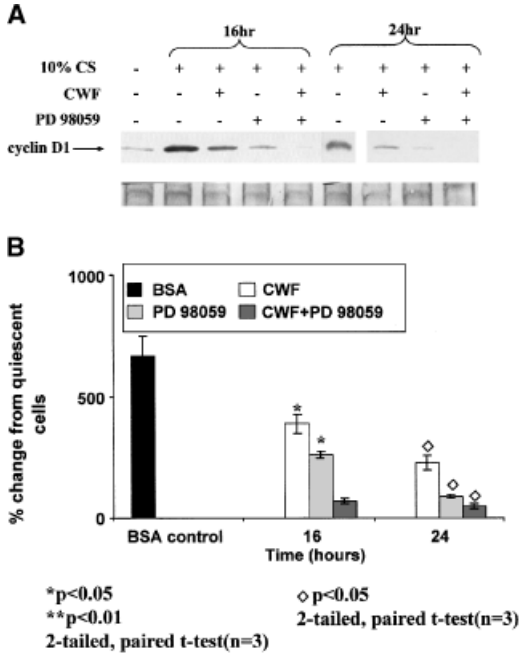

Figure 7
Effects of chronic wound fluid (CWF) and PD 98059 and CWF on the level of cyclin D1. (*A*) Newborn dermal fibroblasts were treated with PD 98059, CWF, or a combination of both for 16 and 24 h, and harvested for immunoblot analysis for detection of cyclin D1. A representative blot for three independent experiments is shown. (*B*) Immunoblot results from three independent experiments were subjected to densitometric analysis and values were averaged and statistical analysis was performed for each time point to compare cyclin D1 levels among treatment groups. The two-tailed paired Student's *t* test showed that mitogen-activated protein kinase kinase inhibition alone significantly blocked serum-induced increase in the level of cyclin D1 ($p < 0.05$–$p < 0.01$), mimicking the effect of CWF ($p < 0.05$–$p < 0.01$). Treatment with both CWF and PD 98059 produced an additive effect, significantly downregulating the level of cyclin D1 ($p < 0.01$), even below the level in quiescent cells.

As expected, serum stimulation caused an increase in the level of ppRb that was inhibited by CWF at the time points examined (Fig 8*A*, *lanes 1–3* and *6*). Similarly, PD 98059 suppressed the level of ppRb at both time points (Fig 8*A*, *lanes 3*, *4*, *6*, and *7*). Results showed that MEK inhibition alone significantly blocked serum-induced increase in the level of ppRb ($p < 0.05$) (Figs 8*A* and *B*), mimicking the effects of CWF ($p < 0.05$) (Figs 8*A* and *B*). Moreover, treatment with both CWF and PD 98059 produced an additive effect, more significantly downregulating the level of ppRb ($p < 0.01$) at both time points (Figs 8*A* and *B*).

Discussion

CWF has been shown to specifically inhibit proliferation of dermal fibroblasts and endothelial cells (Bucalo *et al*, 1993), thus retarding the healing process, whereas acute wound fluid was shown to stimulate proliferation of fibroblasts and endothelial cells (Greenburg and Hunt, 1978; Jalkanen *et al*, 1983; Katz *et al*, 1991). Our results, performed in paired cultures, confirmed the opposing effects of CWF and acute wound fluid on the proliferation of fibroblasts. These results further demonstrate the critical roles that the microenvironment of wounds such as wound fluid may play during the healing process. The molecular mechanisms through which CWF exerts its inhibitory effects on the proliferation of various cell types are, however, not well understood. In this study, we investigated the role of a Ras-dependent signaling pathway in mediating the growth-inhibitory effects of CWF.

Our results show that CWF significantly downregulated the level of active Ras in serum stimulated NbFb. It is well established that activation of the Ras-dependent Raf/MEK pathway is responsible for cyclin D1 upregulation in response to mitogenic stimulation (Filmus *et al*, 1994; Liu *et al*, 1995; Arber *et al*, 1996; Winston *et al*, 1996; Aktas *et al*, 1997; Fan and Bertino, 1997; Kawada *et al*, 1997), and the ability to induce Rb phosphorylation through cyclin D/CDK4-dependent kinases appears to be a major part of the link between Ras and the cell-cycle machinery. Therefore a Ras-dependent pathway may be the major effector pathway mediating the effects of CWF in downregulating the levels of ppRb and cyclin D1.

The involvement of Ras in mediating CWF effects is further confirmed by the results of experiments expressing constitutively active Ras38V in NbFb. The presence of constitutively active Ras abrogated the CWF-induced downregulation of both cyclin D1 and ppRb in response to serum stimulation. Ras activation can result in the activation of

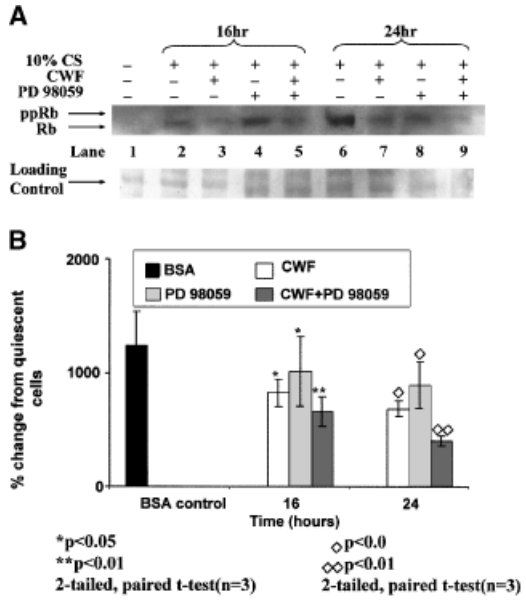

Figure 8
Effects of chronic wound fluid (CWF) and PD 98059 on the level of newborn dermal fibroblasts (ppRb). (*A*) Newborn dermal fibroblasts were treated with PD 98059 (10 μM), CWF (125 μg per mL), or a combination of both for 16 and 24 h, and harvested for immunoblot analysis for detection of retinoblastoma tumor-suppressor gene (Rb). A representative blot for three independent experiments is shown. (*B*) Immunoblot results from three independent experiments were subjected to densitometric analysis and values were averaged and statistical analysis was performed for each time point to compare ppRb levels among treatment groups. The two-tailed paired Student's *t* test showed that mitogen-activated protein kinase kinase inhibition alone significantly blocked serum-induced increase in the level of ppRb ($p < 0.05$), mimicking the effect of CWF ($p < 0.05$). Treatment with both CWF and PD 98059 produced an additive effect, more significantly downregulating the level of ppRb ($p < 0.01$) at both time points.

multiple signaling pathways (Campbell *et al*, 1998; Vojtek and Der, 1998), and the best-characterized Ras-dependent signaling pathway involves activation of MEK (Macdonald *et al*, 1993). Treatment of NbFb with the MEK inhibitor PD 98059, which specifically inhibits the Raf/MEK pathway (Alessi *et al*, 1995; Dudley *et al*, 1995), suppressed the upregulation of both cyclin D1 and ppRb in response to serum stimulation, mimicking the effects of CWF (Figs 7 and 8). Interestingly, PD 98059, at a concentration of 10 μM, caused a greater reduction in the level of cyclin D1 than CWF, measured in parallel. The suppressive effect of PD 98059 on the level of ppRb was, however, less than that of CWF. This further confirms our previous reports that the effects of CWF on ppRb involve not only cyclin D1, but also $p21^{Cip1/Waf1}$ (Seah *et al*, 2001). At a higher PD 98059 concentration (20 μM), serum induction of cyclin D1 was completely blocked (data not shown). Concomitant treatment of NbFb with both CWF and PD 98059 produced additive effects on cyclin D1 and ppRb downregulation in response to serum stimulation. This additive effect suggests that CWF may be exerting its effects on cyclin D1 and ppRb primarily through a Raf/MEK, Ras-dependent pathway. It would be interesting to determine whether this pathway can be completely blocked using higher concentrations of CWF. Moreover, it has been previously reported that the extent of growth inhibition by CWF varies among patients (Phillips *et al*, 1998). It may be that CWF from ulcers of longer duration may be more potent in suppressing Ras activity, thus almost completely inhibiting the important pathway required to induce Rb phosphorylation.

Results presented here suggest that CWF exerts it effects on cell-cycle-regulatory proteins, in part, by downregulating active Ras. The mechanism through which CWF is able to downregulate the level of active Ras still remains to be investigated. It is possible that factors in CWF compete with growth factors for receptor binding sites, resulting in decreased receptor tyrosine kinase activation of Ras. Alternatively, factor(s) in CWF may upregulate molecules that interfere with the GTP-loading action of Ras, resulting in reduced levels of active Ras. The possibility that factor(s) in CWF interfere with MEK phosphorylation resulting in diminished MAP kinase pathway activation also cannot be ruled out.

Experiments described in this report were performed using NbFb. Presumably, fibroblasts exposed to CWF *in vivo* would be adult dermal fibroblasts, and it is not clear whether CWF would have a similar effect on adult dermal fibroblasts. When CWF was added to primary human dermal fibroblasts cultured from biopsies taken from normal adult skin, ages ranging from 40 to 70 y, CWF similarly suppressed the proliferation of these adult fibroblasts (Park *et al*, 1998). These results suggest that effects of CWF on adult dermal fibroblasts would be similar to those on NbFb.

It is yet to be determined what factor(s) in CWF is responsible for the growth inhibitory activity. Our previous studies demonstrated that CWF inhibits growth of NbFb in a concentration-dependent fashion (Phillips *et al*, 1998). When 100, 250, 500, and 1000 μg protein of CWF was added to paired cultures of NbFb, 500 μg protein per plate showed about 60%–75% inhibition whereas 1000 μg protein per plate inhibited the growth of NbFb by greater than 90% (Phillips *et al*, 1998). Preliminary biochemical analysis using column chromatograph fractionations suggested that the growth inhibitory activity was highest in fractions that contain molecules with molecular weights less than 30 kDa (Bucalo *et al*, 1993). Among them, tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) were the best suggested candidates that may inhibit proliferation of NbFb. Neutralizing TNF-α activity in CWF with a specific monoclonal antibody against TNF-α did, however, not reduce growth inhibitory activity in CWF (Mendez *et al*, 1999). TGF-β, when added alone to the dermal NbFb, suppressed proliferation of these cells (Mendez *et al*, 1999) but neutralizing its activity in CWF using a specific monoclonal antibody did not reduce the growth inhibitory activity (Mendez *et al*, 1999). These results suggest that neither TNF-α nor TNF-β present in CWF play a role in suppressing the growth of dermal NbFb.

CWF was reported to contain an excess level of proteases (Wysocki and Grinnell, 1990), resulting in excess degradation of fibronectins (Wysocki and Grinnell, 1990). MMP-2 and MMP-9 were at least 5-fold higher in CWF when compared with acute wound fluid (Wysocki *et al*, 1993). Furthermore, TIMP-1 is reduced in CWF (Howard *et al*, 1994). Pro-inflammatory cytokine levels such as TNF-α and interleukin (IL)-1β were about 100-fold higher in CWF than in mastectomy fluid (Mast and Schultz, 1996), whereas

the levels of IL-8 were similar in CWF and acute wound fluid (Mast and Schultz, 1996). How the changes in the overall level of cytokines effect impaired healing are, however, yet to be elucidated.

Our results show that CWF affects a definable pathway leading to inhibition of cellular proliferation, even in the presence of serum stimulation. It is therefore unlikely that a lack of mitogenic growth factors is responsible for the inhibitory effect of CWF. This is consistent with a previous study showing no significant differences in the levels of growth factors or cytokines between healing and non-healing chronic venous ulcers (Harris *et al*, 1995). Moreover, clinical studies involving the external application of growth factors to chronic venous ulcers have failed to show consistent efficiency. The presence of specific growth inhibitory factor(s) may be responsible for the inconsistent clinical outcomes with novel treatment modalities such as growth factor therapy and skin equivalent allografts. Since most known growth factors stimulate mitogenesis through a Ras-dependent Raf/MEK pathway, the use of growth factor therapy and living skin equivalents to deliver mitogenic growth factors to enhance wound cell proliferation is destined for poor outcomes. Perhaps use of pharmacologic substances that activate alternative mitogenic pathways would offer better treatment options. Understanding the underlying molecular mechanisms responsible for the impaired healing seen in chronic wounds would greatly enhance development of more effective therapies. The findings reported here expand on studies that have demonstrated the ability of CWF to inhibit dermal fibroblast proliferation, and further elucidate the molecular mechanisms through which CWF is able to exert its growth inhibitory effects. These results may, in the future, allow development of more effective therapies for the treatment of chronic wounds.

Materials and Methods

Fibroblast isolation and culture NbFb were cultured as previously described (Gilchrest, 1980). Briefly, newborn foreskins were treated for 5 min in a povidine–iodine bath, followed by a 5 min 70% ethanol bath. Biopsies were then placed in a 1 mg per mL trypsin solution overnight at 4°C to dissociate the dermis from the epidermis and adipose tissues. Isolated dermal tissue was then cut into 1–2 mm fragments and placed in etched plastic culture plates. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine CS, devoid of any antibiotic or antimycotic agents. Second or third passage cells were used in all the experiments.

Collection of chronic and acute wound fluids CWF and acute wound fluid from each patient was collected and processed separately and was not combined for any experiments. To collect CWF, patients with documented chronic venous ulcers, recruited with the approval of the Institutional Review Board at Boston Medical Center, were treated with Allevyn foam dressings (Smith & Nephew, La Jolla, California) for 24–48 h. Patients with infected ulcers or malignancies were excluded. CWF was then extracted from the foam dressings through sterile syringes with 20-gauge stainless-steel needles. The collected CWF was immediately diluted (1:10) with DMEM containing the protease inhibitors Aprotinin (2 μg per mL) and phenylmethylsulfonyl fluoride (10 μg per mL). Large debris was removed by centrifugation at 14,000 × *g* for 15 min. The CWF was then stored at −70°C until use. Just prior to addition to cell cultures, the CWF was filtered through a 0.4 μm syringe filter to remove bacteria. Subsequently, protein concentration expressed in μg per μL was determined. In order to avoid possible contamination of CWF among the patients each Allevyn foam dressing was processed separately. Then, CWF collected from each patient was used independently in each experiment. To collect acute wound fluid, gauze dressings were placed immediately after a Moh's surgery for 10–30 min. The gauze dressings were then removed and the acute wound fluid was extracted and processed employing the same procedures used for CWF as described above. The study was conducted according to the Declaration of Helsinki Principles and the Institutional Review Board at Boston Medical Center approved the studies. Informed consent signatures were excused by the committee because otherwise discarded wound dressings were used.

Cell synchronization NbFb were plated at 100,000 cells per 60 mm dish in DMEM containing 10% CS and allowed to settle overnight. Cells were then serum starved in DMEM with 0.1% CS for 72 h to synchronize them into G0. Seventy-two hours serum starvation of NbFb has previously been shown to induce quiescence in at least 90% of the cells (Yaar *et al*, 1990).

Transient transfections *Escherichia coli* bacteria containing myc-tagged (9E10) Ras38V cDNA were obtained from Dr Alan Hall (London, UK). Quiescent NbFb plated at 1×10^6 cells per 100 mm dish were stimulated to initiate DNA synthesis with 10% CS. After 24 h stimulation, Ras38V cDNA was transiently transfected into NbFb using Lipofectamine Plus reagents (Gibco BRL Life Technologies, Carlsbad, California) according to manufacturer's instructions. Forty hours after transfection, cells were treated with either BSA or CWF (250 μg per mL) for 6–16 h. Maximal expression for transfected Ras38V occurred between 48 and 72 h after transfection (data not shown). Non-transfected NbFb expressing only endogenous Ras, treated with either BSA or CWF (250 μg per mL), were used as control.

MEK inhibition with PD 98059 NbFb plated at 1×10^5 cells per 60 mm culture dish were synchronized into G0 by serum-starvation a described above. Paired cultures were pre-treated for 30 min with 10 μM of the MEK inhibitor PD 98059 (Calbiochem, LaJolla, California) or an equal volume of vehicle alone. PD 98059 [2-(2′amino-3′-methoxyphenyl)-oxanapthalen-4-one] is a highly specific, non-competitive, inhibitor of MEK activity and consequently, it blocks the MAP kinase pathway (Alessi *et al*, 1995; Dudley *et al*, 1995). Furthermore, growth inhibitory effects of PD 98059 are rapidly reversed when the compound is removed from culture media (Alessi *et al*, 1995; Dudley *et al*, 1995). After 30 min pre-treatment, CWF or BSA (250 μg per mL) was added to NbFb in the presence of 10% CS for 16–24 h. Cells were then harvested for immunoblot analysis.

Ras activity assay Quiescent NbFb were treated with either 250 μg per mL CWF or BSA in the presence of 10% CS for 6–16 h and harvested by scraping in a Mg^{2+} lysis buffer (125 mM HEPES, 750 mM NaCl, 5% Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA, 10% glycerol). After protein concentration was determined (Biorad Detergent Compatible Protein Assay, Bio-Rad, Hercules, California), cell lysates were then incubated with a GST–RBD immobilized on glutathione–sepharose (Upstate Biotechnology, Charlottesville, Virginia) for 30 min at 4°C with rocking. Approximately 15 μL of packed GST–RBD beads were used per 15–30 μg of cell lysate protein. After the 30 min incubation, beads were washed in Mg^{2+} lysis buffer. Precipitated protein was released from the beads by boiling the samples in Laemmli sample buffer (Laemmli, 1970). The samples were then subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and precipitated active Ras was detected by immunoblotting. Raf-RBD has a significantly lower affinity for inactive Ras-GDP than for active Ras-GTP, and so only active Ras-GTP is detected under the conditions of this assay (Taylor and Shalloway, 1996).

Protein extraction and immunoblot analysis NbFb were harvested in a RIPA lysis buffer (0.25 M Tris/HCl, 0.75 M NaCl, 2.5% SDS, 0.1% Triton) containing phenylmethylsulfonyl fluoride (1 μM), aprotinin (1 μM), and okadaic acid (1 μM) at 16, 20, 24, and 28 h after treatment. Lysate samples were sonicated briefly and centrifuged at 13,000 × *g* to remove the particulate fraction. Lysate samples was resolved by SDS-PAGE on 5%–12% polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad). The membranes were blocked in 5% non-fat milk and blotted with antibody against Rb (Pharmingen, San Diego, California), cyclin D1 (Pharmingen), and Ras (Tranduction Labs, Lexington, Kentucky). This was followed by incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase. Protein bands were visualized using ECL detection system (Amersham, Piscataway, New Jersey).

FACS analysis Flow cytometry was used to determine whether CWF induces apoptosis in NbFb. Cells were plated at 1×10^6 cells per 100 mm dish, and synchronized into G0 as described above. Cells were then treated with either BSA or CWF (250 μg per mL) for 24 h in the presence of 10% CS. For positive apoptosis controls, NbFb were incubated with 0.1 μM staurosporine (Calbiochem), a potent protein kinase inhibitor capable of inducing a strong apoptotic response in normal human fibroblasts (Tepper *et al*, 2000). Parallel cultures of NbFb were serum starved in DMEM with 0.1% CS for 24 h as an additional control. Single color flow cytometric analysis of DNA content by PI staining was carried out. After the various treatments, cells were detached with 1 μg per mL trypsin/1 mM EDTA, pelleted, washed, and fixed in 70% ethanol. Fixed cells were washed again with ice-cold phosphate-buffered saline (PBS) before incubation in 8 μg per mL RNAse A (Sigma, St Louis, Missouri) and 18 μg per mL PI (Sigma) at 37°C for 30 min. Cells were analyzed with a FACScan (Becton Dickinson, Sparks, Maryland). Cell-cycle analysis of ungated data and determination of the percentage of cells in G0/G1, S, and G2/M were calculated using CELLQuest software.

TUNEL apoptosis assay Paired cultures of NbFb were plated at 1×10^{-6} cells per 100 mm dish and 24 h after plating, cells were treated with BSA or CWF (250 μg protein per mL media). Twenty to 24 h after the treatments, cells were trypsinized, and then fixed in 2% paraformaldehyde in PBS (pH = 7.4) for 15–20 min at room temperature. Cell membranes were then permeabilized by treating the cells with 0.1% Triton X-100 in 0.1 sodium citrate. Cells were then further processed using *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. The level of fluorescence in each treatment group was determined using FACS analysis.

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