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Co-ordination between localized wound-induced Ca^{2+} signals and pre-wound serum signals is required for proliferation after mechanical injury

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Abstract. The signals which initiate proliferation of endothelial cells after injury are important for selective blood vessel growth during wound healing or tumour growth. Upon mechanically wounding quiescent cells, a transient $[Ca^{2+}]_i$ increase was induced in cells at the wound edge. These same cells proliferated 18–24 h post wounding, as measured by bromodeoxyuridine incorporation. The localized Ca^{2+} signal was required specifically during wounding since blocking Ca^{2+} influx reduced proliferation by 40–50%. Proliferation also required serum since starvation reduced proliferation by 80%. Serum-starved cells proliferated if briefly primed with serum prior to wounding. The signals derived from serum and $[Ca^{2+}]_i$ combined at least additively to induce proliferation. Therefore, serum priming followed by a single, transient Ca^{2+} signal induced by mechanical injury must occur in a temporally and spatially regulated manner for normal proliferation. Co-ordination between signalling cascades induced by growth factors and release from contact inhibition might be obligatory for localized re-endothelialization after injury.

Endothelial cell proliferation contributes to the closure of large wounds in the endothelium and is also central to neovascularization of the site of tissue injury. The rate and extent at which re-endothelialization occurs in a denuded zone determines the extent and duration of the initial mechanical injury (Madri *et al.* 1988). Strict regulation of cellular proliferation is therefore of immense importance to normal processes of healing and to the prevention of many pathological conditions like atherosclerosis, diabetic retinopathy and restenosis. Although the process of proliferation has been well studied, the signals that regulate the induction of endothelial cell proliferation after mechanical injury have not. In particular, interaction between signalling pathways and the spatial and temporal patterns of signalling mechanism that might trigger cell proliferation have not been well explored. The study of endothelial cells *in vitro* allows us to elucidate the signals which are induced upon release from contact inhibition to selectively stimulate cellular proliferation responses (Gotlieb *et al.* 1987).

Earlier investigations have shown that, shortly after wounding a confluent monolayer, motility is induced in cells at the edge of a wound. If the denuded zone is too large to be

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completely closed by cell movement, cellular proliferation is initiated (Coomber & Gotlieb 1990). This inducation of proliferation upon wounding depends partly on release from contact inhibition. Dulbecco found that serum starvation of 3T3 cells, cultured in media containing 2% serum before wounding, affected the induction of DNA synthesis in cells away from the wound but not cells at the edge of the wound (Dulbecco 1970). Serum is believed to be required for the re-entry of quiescent cells into the G_1 phase of the cell cycle from G_0 (Durham & Walton 1982). A short serum pulse may trigger quiescent serum-starved cells in G_0, to enter the G_1 phase of the cell cycle (Durham & Walton 1982, Pardee 1989). It has also been proposed that serum is required throughout G_1 and passage through late G_1 requires continued signalling by serum (Assoian & Zhu 1997). However, serum was not required for wound-induced proliferation of 3T3 fibroblast cells at the edge of the wound (Dulbecco 1970). This suggests a diversification in the regulation of cell progression through the cell cycle and the mechanical induction of proliferation.

Many studies have also found an important role for Ca^{2+} in cell cycle regulation. Induction of proliferation by serum addition to starved fibroblasts requires elevation of $[Ca^{2+}]_i$ (Wahl & Gruenstein 1993). Calcium is known to activate the re-entry of quiescent cells from G_0 into G_1 by inducing immediate early gene expression and subsequent transcriptional activation of cell cycle dependent proteins like the cyclins (Takuwa *et al.* 1995). Another transient Ca^{2+} spike at the G_1/S interphase may promote the phosphorylation of the retinoblastoma protein, Rb, thereby committing cells to DNA synthesis (Berridge 1995). Early studies found that addition of buffercontaining high concentrations of Ca^{2+} to cells stimulates thymocyte as well as hepatocyte proliferation (Whitfield *et al.* 1980). In addition, agonists, which are known to increase Ca^{2+} influx, were also shown to induce hepatocyte and lymphoma cell proliferation (Whitfield *et al.* 1980, Ridefelt *et al.* 1996). We recently found that mechanically wounding cell monolayers releases wound-derived factors that elevate $[Ca^{2+}]_i$ in cells near the wound edge (Sammak *et al.* 1997, Hinman *et al.* 1997). The wound-induced elevation of $[Ca^{2+}]_i$ stimulates cell movement (Sammak *et al.* submitted) and immediate early gene expression (Tran *et al.* 1998). Given the role that $[Ca^{2+}]_i$ has in agonist-stimulated proliferation, we considered whether mechanical injury induced proliferation by Ca^{2+} signalling as well.

Although from earlier studies (Berridge 1995, Kohn *et al.* 1995, Ridefelt *et al.* 1996) we know that both serum and Ca^{2+} are required for proliferation, it is not known how they interact with each other to promote proliferation in the context of normal re-endothelialization after injury. In this study, we elucidated the times at which serum and the rise in $[Ca^{2+}]_i$ may be required and how they may interact with each other to induce proliferation in bovine pulmonary endothelial cells following mechanical injury. We found that the normal induction of proliferation hours after the initial mechanical stimulus requires FBS priming before wounding and a $[Ca^{2+}]_i$ increase during wounding. These two factors behave additively to induce proliferation in serum-starved cells to level greater than that observed in serum-fed cells.

METHODS

Cell culture

Calf pulmonary artery endothelial cells (CPAE 209, line CCL 209), obtained from American Type Culture Collection (ATCC, Rockville, MD) were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco Life Technologies, Grand Island, NY) supplemented with 20% foetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES, Fisher Chemicals, Itasca,

IL) and 1% penicillin/streptomycin (Gibco Life Technologies, Grand Island, NY), pH 7.4 (serum DMEM). Endothelial cells were plated onto glass coverslips (Corning Glass, Corning, NY) and grown to confluency. Upon reaching confluency, cells were either used for experimentation (serum-fed cells) or serum starved in DMEM supplemented with 0.1% bovine serum albumin (BSA, Sigma Chemicals, St Louis, MO), 20 mM HEPES, and 1% penicillin/streptomycin (serum-free DMEM) for 2 days prior to experimentation (starved cells).

$[Ca^{2+}]_i$ measurements using fura-2

Prior to experimentation, serum-fed cells grown on glass coverslips were mounted in dishes with holes to facilitate imaging. $[Ca^{2+}]_i$ measurements using fura-2 AM was done as previously described (Hinman *et al*. 1997, Sammak *et al*. 1997). Briefly cells were exposed to a solution of 2 μM fura-2 AM (Molecular Probes, Eugene, OR) and 0.03% pluronic acid in 1.5 ml Hanks balanced salt solution (HBSS, Gibco Life Technologies, Grand Island, NY) containing 1.3 mM Ca^{2+} without phenol red, supplemented with 20 mM HEPES, pH 7.4. Each coverslip was rinsed once with HBSS immediately before imaging and kept in HBSS under minimal light conditions at room temperature until imaging was done. Cell monolayers were mechanically wounded with a needle which created a denuded zone small enough to be detected within the microscopic field. Wounding was performed in either HBSS buffer, in Ca^{2+}-free HBSS containing 2.5 mM ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA, Sigma, St Louis, MO), a chelator of extracellular Ca^{2+}, or in phosphate-free HBSS containing Ca^{2+} and freshly prepared 100 μM gadolinium (Gd^{3+}, Aldrich, Milwaukee, WI), a Ca^{2+} channel blocker. $[Ca^{2+}]_i$ was measured during wounding by fluorescence ratio imaging using a 20X CF fluor objective on a Nikon Diaphot 300 inverted microscope connected to a Photometrics PXL cooled CCD camera (Tucson, AZ). The camera was controlled by the IPLab Spectrum ratioing program (Signal Analytics, Vienna, VA) on an Apple Power Mac 8500/120 computer as previously described (Hinman *et al*. 1997). Ratios were calibrated *in vitro* using a saturated 10 mM Ca^{2+} solution and a nominal Ca^{2+}-free solution containing 10 mM EGTA (Hinman *et al*. 1997). All Ca^{2+} imaging experiments were performed at 20–25°C. $[Ca^{2+}]_i$ measurements presented are an average of three wounds per condition, an average of 30 cells per wound.

Proliferation assay

Cell proliferation was measured using immunohistochemical detection of bromodeoxyuridine (BrdUrd, Sigma, St Louis, MO). BrdUrd, a thymidine analogue that can be added to the media, is only incorporated into the DNA of actively proliferating cells. All control cell monolayers were wounded in HBSS by replacing the cell media with HBSS for 30 s, then removing half of the cells on the coverslip with a rubber policeman. Half of the cells were removed to insure the induction of proliferation since wounding with a needle would result in a denuded zone small enough for wound closure to occur solely through cell motility (not shown and Coomber & Gotlieb 1990). After a further 1 min and 30 s, the HBSS was replaced with the appropriate media. Eighteen hours after the initial wound stimulus, BrdUrd was added to the media at a final concentration of 1 μM. After 6 h of BrdUrd incorporation, cells were fixed using either a 95:5 mix of ethanol:acetic acid for 30 min or 100% methanol for 6 min at −20°C for more stable fixation. The period from 18 h to 24 h after the initial wound stimulus was determined to be the period of peak proliferation (data not shown). BrdUrd was added specifically during this period of peak proliferation to detect only cells induced to enter the S phase of the cell cycle by the wound stimulus and to avoid high background staining due to low levels of proliferation in unstimulated cells. Immunohistochemistry was

done using a primary mouse antibody to BrdUrd, a peroxidase-conjugated goat anti-mouse IgG secondary antibody, and metal enhanced 3′,3-diaminobenzidine (DAB) as the peroxidase substrate (Sigma, St Louis, MO). After exposure to the DAB solution, cell nuclei with BrdUrd incorporated into the DNA can be viewed as dark black/blue dots under bright field microscopy. Corresponding digital images of the cell monolayers under phase contrast and bright field microscopy were obtained using a 10× phase contrast objective and Cohu video camera mounted on a TMS inverted microscope controlled by the public domain program NIH Image 1.61 (available from ftp://codon.nih.gov/pub/nih-image/nih-image161_ppc.hqx) run on a Power PC 8500 Macintosh. The percentage of cells which incorporated BrdUrd (percentage of proliferating cells) was calculated by dividing the number of BrdUrd incorporated nuclei detected using bright field microscopy by the number of total cell nuclei in the same area detected under phase contrast microscopy. The percentage of proliferating cells in drug-treated experiments were normalized by and compared to the appropriate control (% control). Statistical significance was determined using the Bonferroni *post hoc* test with $P<0.05$. At least $n=3$ replicates were done per treatment.

RESULTS

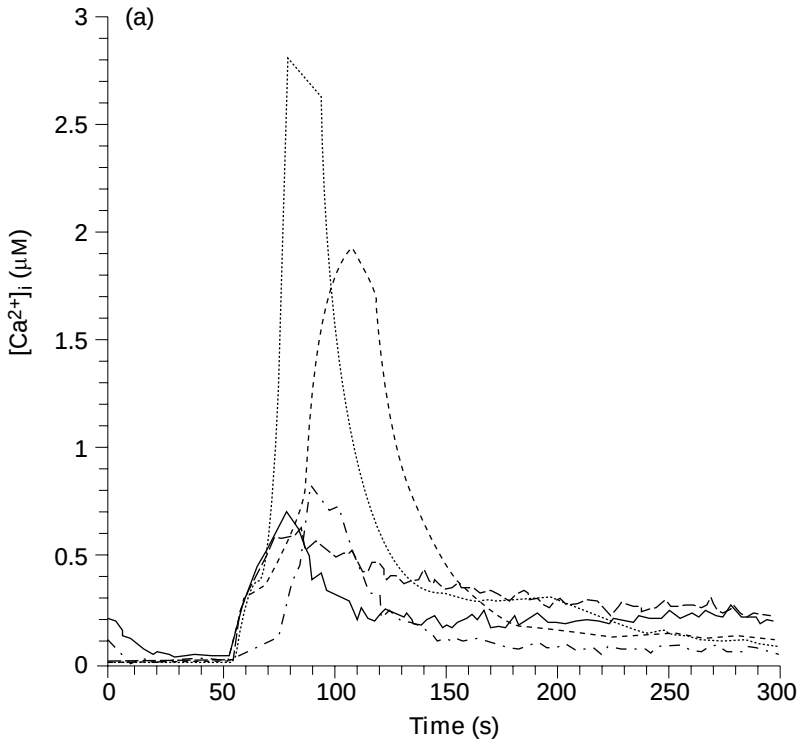
A rise in $[Ca^{2+}]_i$ was induced upon mechanical wounding

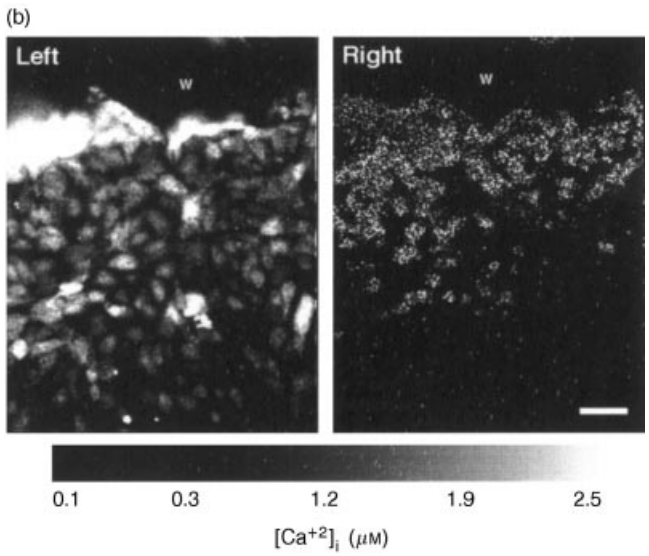
Mechanical injury of confluent endothelial cell monolayers produced a transient rise in $[Ca^{2+}]_i$ in cells remaining next to the wound which propagated 10–12 rows from the wound edge. This elevation was produced by extracellular release of Ca^{2+}-stimulating factors from the wound site (Sammak *et al*. 1997). The rise in $[Ca^{2+}]_i$ reached average peak concentrations of 1.6 ± 0.1 μM (Figure 1a). Serum-starved cells wounded in HBSS exhibited slightly lower, but comparable peak levels of $[Ca^{2+}]_i$ elevation (1.3 ± 0.1 μM, Tran *et al*. 1998). Therefore, upon mechanical wounding, serum-fed cells experience a slightly higher but not statistically different level of $[Ca^{2+}]_i$ increased to that observed in serum-starved cells (Tran *et al*. 1998). Wounding serum-fed cells in 2.5 mM of the extracellular calcium chelator, EGTA or the calcium channel blocker, Gd^{3+}, at 100 μM or 500 μM decreased peak levels of $[Ca^{2+}]_i$ (0.8 ± 0.2 μM, 1.2 ± 0.1 and 0.6 ± 0.3 μM, respectively). In addition, the rise in $[Ca^{2+}]_i$ can be augmented to 2.5 ± 0.2 μM by wounding in the presence of 2.5 mM extracellular Ca^{2+} (Figure 1a). These short-term treatment produced no toxic effects on the cells. Therefore, mechanical injury produced $[Ca^{2+}]_i$ elevations in surviving cells at the wound edge which can be augmented or reduced by altering Ca^{2+} influx.

Induction of proliferation following mechanical injury

Between 18 h and 24 h after the initial wound stimulus, BrdUrd stained cells could be detected as a clear band at the edge of the wound under bright field microscopy (Figure 2a:

Figure 1. Wounding induced an immediate, but transient rise in $[Ca^{2+}]_i$ (a) Ca^{2+} imaging with fura-2 showed a transient increase in $[Ca^{2+}]_i$ when serum-fed cells are wounded. This transient increase can be blocked by using either 500 μM Gd^{3+}, a calcium channel blocker, or 2.5 mM EGTA, a calcium chelator. The $[Ca^{2+}]_i$ rise can also be augmented by wounding in HBSS containing 2.5 mM Ca^{2+}. All peak values were statistically significant from HBSS control values, $P<0.05$. Traces were generated from ≈30 cells 2–3 rows back from the wound edge and are representative of $n\geq3$ wounds per treatment. (b) Representative intensity modulated image of fura-2 loaded cells at the wound edge (w) 6 s post wounding (left). Cells experiencing the peak increase in $[Ca^{2+}]_i$ are located in the first 10 rows near the edge of the wound (Isobestic image, right). Bar in right panel denotes 50 μm. ----, HBSS; —·—·—, 100 μM Gd^{3+}; ———, 500 μM Gd^{3+}; ———, 2.5 mM EGTA; ·····, 2.5 mM Ca^{2+}.


(a)
3
2.5
2
1.5
1
0.5
0
[Ca2+]i (μM)
0
50
100
150
200
250
300
Time (s)


(b)
Left
w
Right
w
0.1
0.3
1.2
1.9
2.5
[Ca+2]i (μM)

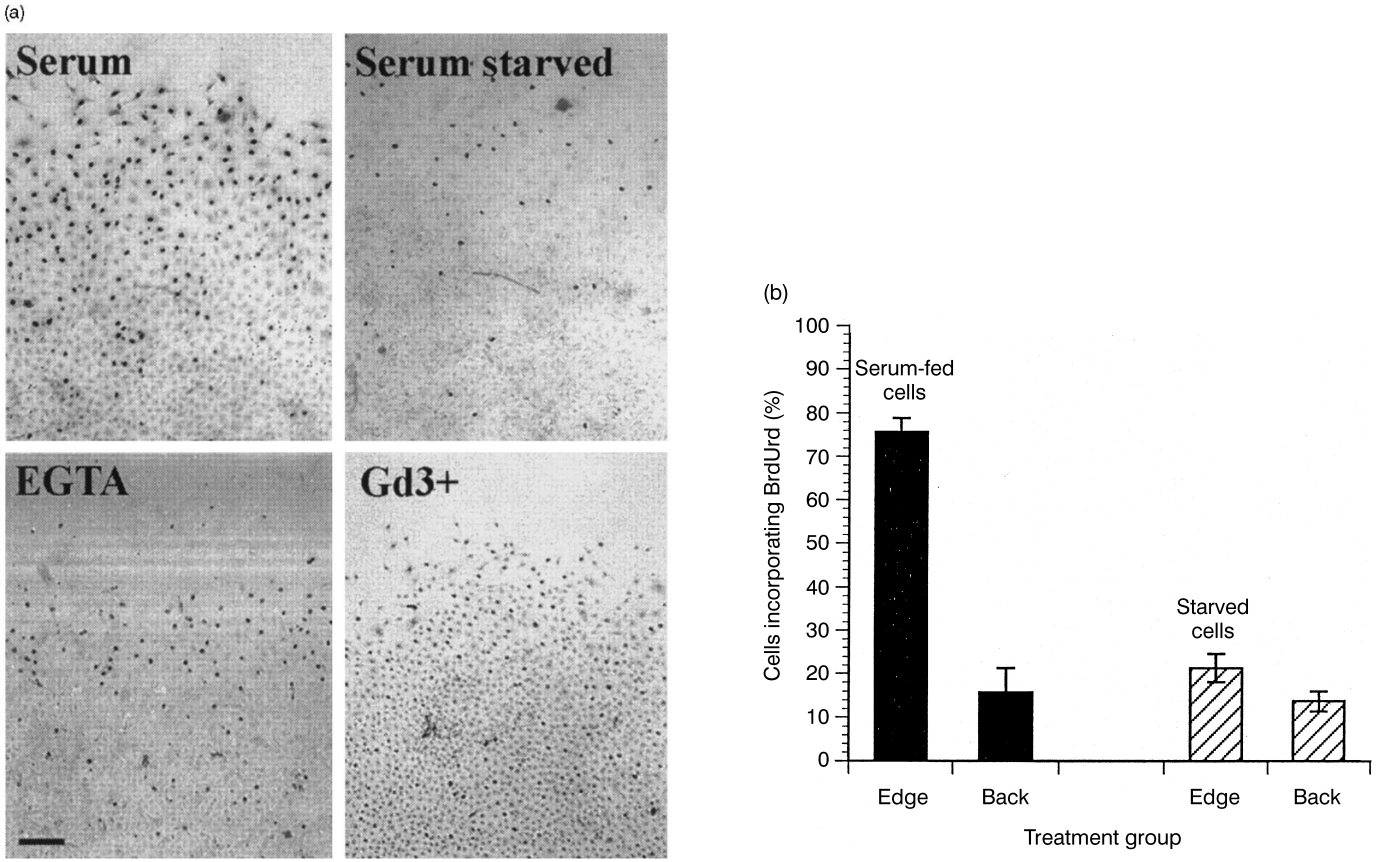


Figure 2. Serum was necessary for wound-induced proliferation. (a) Bright field microscopy of BrdUrd-incorporated serum-fed control cells and serum-starved cells wounded in HBSS or serum-fed cells wounded in the presence of 500 μM Gd^{3+} or 2.5 mM EGTA. Representative of cells from at least 3 experiments. Bar in EGTA panel denotes 100 μm. (b) Comparison of the percentage of proliferating cells both at the edge of the wound and >10 rows back, in serum-fed (black bars) vs. serum-starved cultures (hatched bars). Serum-starved cells proliferated at levels comparable to serum-fed cells within the monolayer that were distant from wounds ($n \geq 5$).

Serum). Cells experiencing the wound-induced rise in $[Ca^{2+}]_i$ (Figure 1b) are the same cells in the band of proliferation, detected with BrdUrd staining, within the first 10 rows from the wound edge (Figure 2a: serum). Seventy-five per cent of the cells in the first 10–15 rows near the edge of the wound proliferated in serum control coverslips during the 6 h interval starting 18 h postwounding (Figure 2b). Fifteen or more rows further back from the wound edge, only 15% of the cells proliferated (Figure 2b). This level of proliferation was comparable to the level of proliferation in non-wounded confluent monolayers (15–20%). After mechanical injury, proliferation, as detected by BrdUrd labelling, was induced selectively in cells near the edge of the denuded zone.

Serum and the wound-induced rise in $[Ca^{2+}]_i$ are both required for proliferation

To determine if serum was necessary for the induction of proliferation, cells were serum starved for 2 days in serum-free DMEM. The percentage of proliferating cells at the edge of the wound in serum-starved coverslips decreased to 20% of the serum control (Figure 2b). Although there was no apparent band of proliferating cells at the edge of these wounds (Figure 2a: serum starved), this percentage of proliferating cells was still significantly above the percentage found in cells located further back from the wound (Figure 2b). The percentage of proliferating cells for cell populations further back from the wound edge did not differ between serum-fed and serum-starved cultures (Figure 2b).

Since the wound-induced rise in $[Ca^{2+}]_i$ occurred in the same cells that were induced to proliferate hours after wounding, we also evaluated whether this Ca^{2+} rise was required for the induction of proliferation. When confluent cell monolayers were wounded in HBSS containing Gd^{3+} or EGTA to reduce Ca^{2+} influx, the percentage of proliferating cells at the edge of the wound decreased (Figure 2a: Gd^{3+} and EGTA). Gd^{3+} at 500 μM final concentration was used in these studies because it decreased the Ca^{2+} rise to the same degree as 2.5 mM EGTA did in cells cultured in DMEM containing 20% FBS. Wounding in either phosphate-free HBSS containing 500 μM Gd^{3+} or Ca^{2+}-free HBSS containing 2.5 mM EGTA decreased the level of proliferation to 50–60% of the serum control (Figure 3). Treatment with either Gd^{3+} or EGTA did not have non-specific effects since 2 min treatment with either drug 20 min before or after wounding did not have any effect on the percentage of proliferating cells (Figure 3). These results show that the presence of serum and a rise in $[Ca^{2+}]_i$ induced by wounding are both necessary for the induction of proliferation after mechanical wounding.

Temporal requirement for serum stimulation

To elucidate the interactions between the signals induced by serum and by wounding, we first determined whether the timing of these signals was important. Serum is required at different times for cells to pass through specific cell cycle checkpoints. A short serum pulse may trigger quiescent serum-starved cells in G_0 to enter the G_1 phase of the cell cycle (Durham *et al.* 1982, Pardee 1989). It has also been proposed that serum is required throughout G1 and passage through late G1 requires continued signalling by serum (Assoian & Zhu 1997). To determine if a single pulse of serum was sufficient to return proliferation levels in serum-starved cells back to serum control levels, serum-starved cells were exposed to a serum pulse 1.5 h prior to wounding by replacing the starvation media with serum media for 30 min. Cells were then returned to starvation media for an hour before wounding in HBSS. The 30 min FBS priming pulse increased proliferation levels in serum-starved cells to those cells fed continuously with serum (Figure 4). To determine if serum stimulation during or post wounding was sufficient to induce normal levels of proliferation post wounding, serum-free

DMEM was replaced with serum DMEM for 30 min either around the time of wounding (from 15 min before wounding to 15 min post wounding) or at 1 h after wounding. Neither treatment increased proliferation levels over serum-starved levels (Figure 4). Therefore, cells need to be serum primed 1 h before wounding in order for proliferation to be induced in cells at the edge of the wound. Release from contact inhibition alone was not sufficient unless quiescent cells in G_0 have been primed to re-enter G_1 by addition of serum. Serum signalling late in G_1 was not required.

Temporal requirement for the $[Ca^{2+}]_i$ rise

The temporal requirement for the wound-induced Ca^{2+} signal was also evaluated by blocking the wound-induced $[Ca^{2+}]_i$ elevations and artifically elevating $[Ca^{2+}]_i$ at selected times. Serum-fed cells were wounded in either Ca^{2+}-free HBSS containing 2.5 mM EGTA or phosphate-free HBSS containing 100 μM Gd^{3+}. Immediately following the 2 min wounding

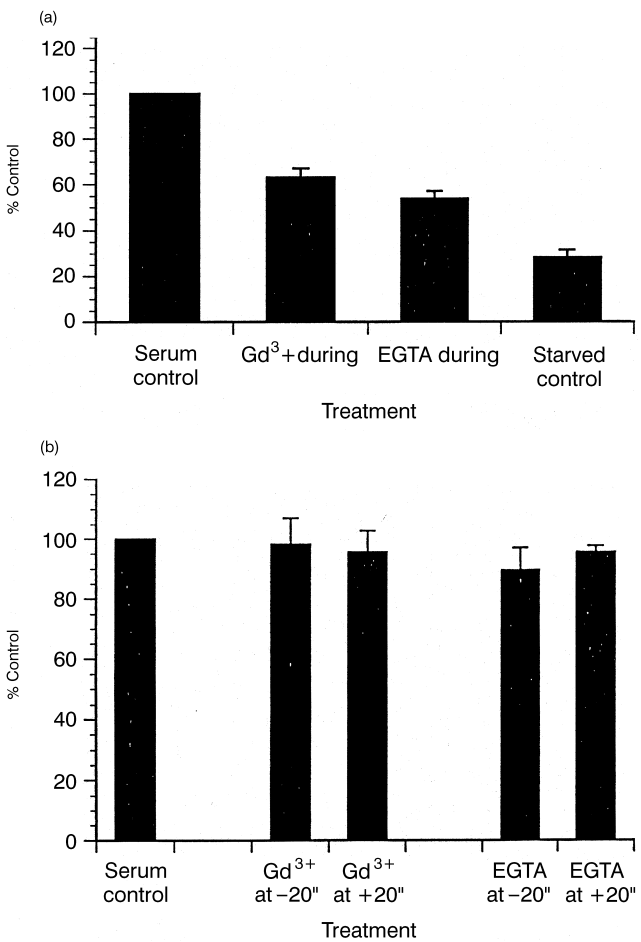


Figure 3. The rise in $[Ca^{2+}]_i$ was required specifically during wounding for normal induction of cell proliferation. (a) Decreasing the $[Ca^{2+}]_i$ rise by wounding in the presence of 500 μM Gd^{3+} or 2.5 mM EGTA also resulted in a decrease in the percentage of proliferating cells. (b) Neither 500 μM Gd^{3+} or 2.5 mM EGTA treatment had non-specific or toxic effects, since pre or post wound treatment with drug did not affect the percentage of proliferating cells ($n \geq 3$).

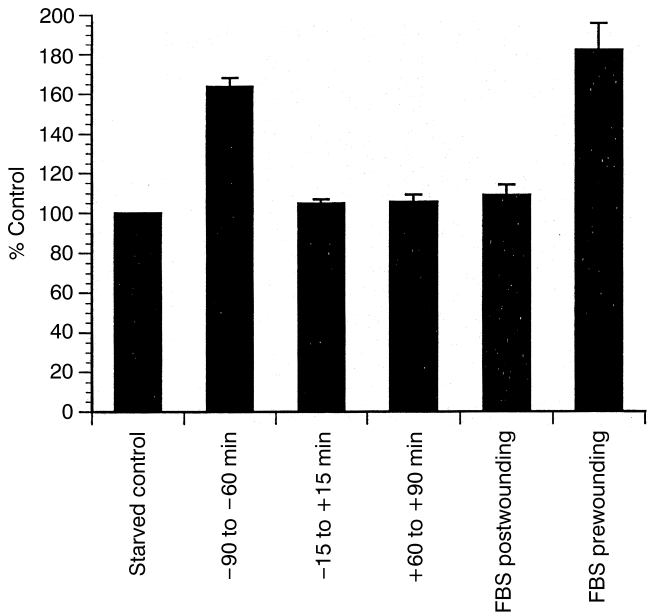


Figure 4. Serum priming was required specifically before wounding. Serum-starved cells primed with a 30 min pulse of serum at least 1.5 h before wounding proliferated at serum control levels. Exposing cells to a 30 min serum pulse during the time of wounding or 1.5 h after wounding did not increase proliferation above starved control levels ($n \geq 3$).

time, cells were washed with HBSS and then exposed to 10 μM of the calcium ionophore, ionomycin, in HBSS containing 2.5 mM Ca^{2+} for 2 min. Cells exposed to the ionomycin/calcium solution without wounding experienced a Ca^{2+} rise comparable to that observed with wounding (data not shown). The decrease in proliferation levels observed after wounding in the presence of EGTA was circumvented by ionomycin/calcium treatment immediately after wounding (Figure 5). However, treatment with the ionomycin/calcium solution 20 min pre or post wounding in EGTA did not rescue the inhibition of proliferation by either Ca^{2+} blocking agent (Figure 5). Similar results were observed for cells wounded in buffer containing 500 μM Gd^{3+}. An ionophore-induced elevation in $[Ca^{2+}]_i$ was sufficient to reverse the negative effects of EGTA or Gd^{3+} on proliferation, only if the ionophore was applied immediately, and not before or after wounding. Cells at wound edges that experienced serum and mistimed $[Ca^{2+}]_i$ elevations were unable to proliferate. Therefore, the induction of a $[Ca^{2+}]_i$ elevation was not sufficient unless it was timed properly. It is possible that Ca^{2+} signals were co-ordinated with additional wound-induced events that have yet to be specified.

The rise in $[Ca^{2+}]_i$ induced by wounding was necessary but not sufficient for the induction of proliferation

Since calcium was specifically required during the time of wounding, cells were wounded in HBSS buffer containing 2.5 mM Ca^{2+} to determine whether increasing the rise in $[Ca^{2+}]_i$ during wounding could increase the percentage of proliferating cells above control levels. Wounding in HBSS containing high levels of extracellular Ca^{2+} increased the percentage of proliferating cells 24% over the serum control (Figure 6a). Exposure of cells to high Ca^{2+}

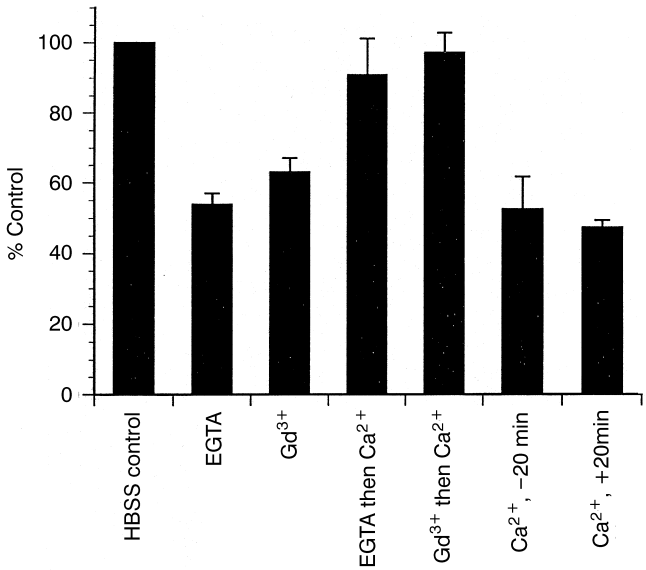


Figure 5. The wound-induced $[Ca^{2+}]_i$ was specifically required during wounding. 1.5 min after wounding in either Gd^{3+} or EGTA, an artificial rise in $[Ca^{2+}]_i$ was induced by the addition of 10 μM ionomycin in HBSS containing 2.5 mM Ca^{2+}. The percentage of proliferating cells in populations exposed to the artificial Ca^{2+} signal was significantly greater than in cells wounded in either Gd^{3+} or EGTA ($n \geq 3$). However, addition of the ionomycin/Ca^{2+} solution for 2 min at 20 min pre or post wounding did not increase the percentage of proliferating cells of Gd^{3+} or EGTA wounded cells to normal ($n \geq 6$).

buffer without wounding did not significantly increase the percentage of proliferating cells above baseline levels (data not shown). Wounding in the presence of high extracellular Ca^{2+}, however, was not sufficient to increase proliferation levels of serum-starved cells to levels comparable to that observed in serum-fed cells. Higher levels of $[Ca^{2+}]_i$ induction increased the percentage of proliferating cells in starved cells by only 20% (Figure 6b) not the 50% required to bridge the gap between serum-fed and serum-starved cells. A minimal rise in $[Ca^{2+}]_i$ was necessary, however, for the induction of proliferation in serum-starved coverslips since wounding in the presence of EGTA or Gd^{3+} decreased the percentage of proliferating cells further to baseline non-wounded levels (Figure 6b). Therefore, the rise in $[Ca^{2+}]_i$ was not sufficient to initiate proliferation hours after the initial wound stimulus. However, it was necessary for normal induction of proliferation and was rate-limiting since wounding in the presence of high extracellular Ca^{2+} concentrations increased proliferation above normal levels.

Additivity of serum priming and the wound-induced $[Ca^{2+}]_i$ signal

To evaluate whether the temporally regulated interaction between serum and $[Ca^{2+}]_i$ was synergistic or additive in the induction of proliferation, serum-fed cells were wounded in Ca^{2+}-free HBSS containing 2.5 mM EGTA to decrease the wound-induced rise in $[Ca^{2+}]_i$. Alternatively, to expose cells to normal wound-induced increases in $[Ca^{2+}]_i$ without serum stimulation, serum-starved cells were wounded in HBSS. The sum of the percentage of proliferating cells observed in cell populations exposed to serum, but not the Ca^{2+} signal, and those experiencing the Ca^{2+} signal without pre exposure to serum was somewhat less than the percentage of proliferating cells in serum control cells where both signals are present

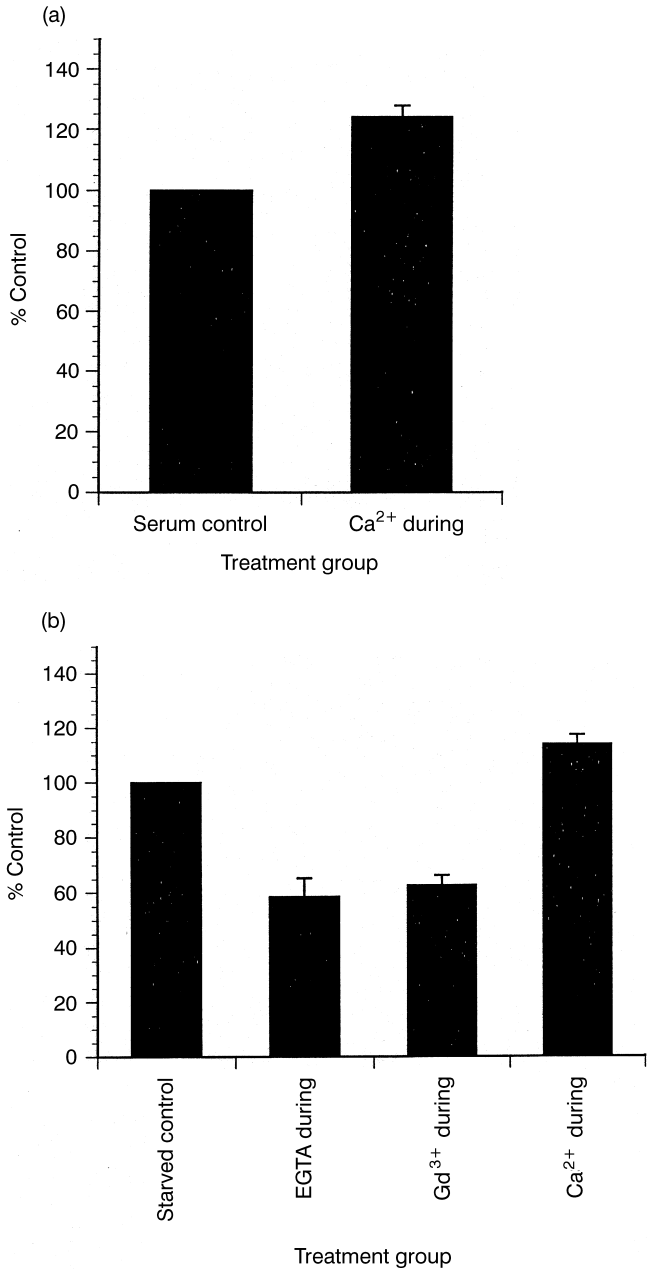


Figure 6. Ca^{2+} was necessary, but not sufficient for the induction of normal proliferation levels without serum. (a) Wounding serum-fed cells in HBSS containing 2.5 mM Ca^{2+} increased the percentage of proliferating cells ($n=3$). (b) Wounding starved cells in HBSS containing 2.5 mM Ca^{2+} was not sufficient to induce proliferation back up to serum control levels ($n=3$). Wounding in Ca^{2+}-free HBSS containing 2.5 mM EGTA or phosphate-free HBSS containing 100 μM Gd^{3+} decreased the percentage of proliferating cells in serum-starved cells further to baseline non-wounded levels ($n \geq 3$).

(Figure 7). Therefore, in addition to release from contact inhibition, a sequence of events involving serum priming of cells followed by a wound-induced $[Ca^{2+}]_i$ signal was requiredto stimulate quiescent cells at a wound edge to re-enter and progress through the cell cycle. The serum-dependent and the Ca^{2+}-dependent signals acted at least additively to induce normal levels of proliferation following mechanical wounding.

DISCUSSION

The process of wound healing involves a complex series of events which determine the extent and duration of the injury. Among the key events in the healing of large wounds is the process of cellular proliferation. Early studies determined that both release from contact inhibition and serum were important for the induction of proliferation (Dulbecco 1970, Assoian & Zhu 1997). The question of the necessity of serum is a highly interesting one, especially in the cases of mechanical injury and tumour cell growth. It is also important to consider that endothelial cells may have different requirements for serum based on their physiological roles in the response to injury and during the process of healing.

Ca^{2+} is another signal with an essential role in the regulation of the cell cycle. Early studies have demonstrated that an agonist-induced increase in $[Ca^{2+}]_i$ is required for the induction of proliferation in normal serum-fed cells, but not in serum-fed cancer cells

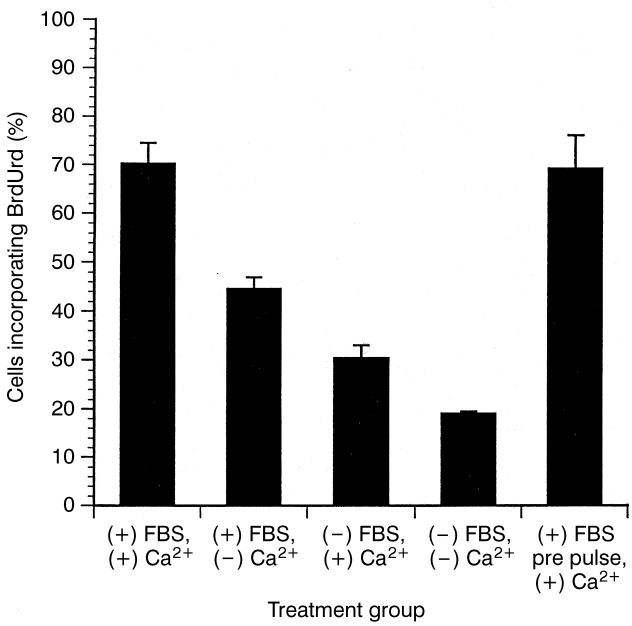


Figure 7. Serum priming and the wound-induced $[Ca^{2+}]_i$ rise acted additively. Wounding in the presence of EGTA blocked the percentage of proliferating cells in serum-fed cells by 50% from the control. Removing serum by starvation had a similar negative effect (80% reduction) to blockage of the $[Ca^{2+}]_i$ rise. When both serum and the $[Ca^{2+}]_i$ signal was removed, the percentage of proliferating cells decreased to baseline levels. Cells which were exposed to a 30 min pulse of serum before wounding and experienced a normal increase in $[Ca^{2+}]_i$ upon wounding experienced proliferation levels comparable to that observed in the normal serum-fed control cells. The effect of serum priming and the wound-induced $[Ca^{2+}]_i$ rise was therefore at least additive ($n \geq 3$).

(Durham *et al.* 1982). To our knowledge, however, the same questions have not been explored in serum-starved normal cells. Therefore, the question remains whether the distinct signalling pathways involving either serum or Ca^{2+} are both required and when they must occur for the induction of normal proliferation in quiescent endothelial cells.

We found that DNA synthesis as measured by BrdUrd labelling was induced to peak levels 18 h after mechanical removal of cells from half of the coverslip. Wound-induced proliferation was limited to cells in approximately the first 15 rows or so at the edge of the wound. These cells have also spread and moved more than cells further into the monolayer. This correlation confirmed previous investigators that cell motility and proliferation are connected (Gotlieb *et al.* 1987, Coomber & Gotlieb 1990). The greater proliferative response in cells at the wound edge may be due to the changes in cell shape which results from the movement of these cells into the denuded zone (Coomber & Gotlieb 1990). Both changes in cell shape and adhesion are believed to be necessary for the induction of proliferation (Assoian *et al.* 1997) and may involve changes in cell–cell contact points like gap junctions. However, since the expression of connexin 43, the major gap junction protein in endothelial cells, does not increase in SMC cells upon mechanical injury of rat iliac arteries (Polacek *et al.* 1997), the difference in the percentage of proliferating cells may be simply due to the greater extent of release from contact inhibition observed in cells near the wound edge. Further studies need to be done to specifically determine if gap junctions, adherens junctions or other routes of signal transduction through the endothelial monolayer are responsible for the difference. Interestingly, we also found that the rows of cells labelled by BrdUrd hours after the initial injury are the same ones which experience a transient rise in $[Ca^{2+}]_i$, immediately upon wounding, which reach peak levels of about 1.3 μM. The spatial correlation suggests that essential chemical signals important for proliferation may be derived from the wound after mechanical injury (Sammak *et al.* 1997).

The induction of endothelial cell proliferation at the edge of the wound was dependent on the presence of serum growth factors since the percentage of proliferating cells induced in serum-starved cells was only about 20% of that observed in serum-fed cultures. The contradiction between these results and those reported earlier by Dulbecco (1970) may be due to the difference in starvation method or to differences in cell response (endothelium vs. fibroblast). Unlike short starvation periods in low serum concentrations, starvation in serum-free media for extended periods may result in the expression of genes like the serum deprivation response gene (*sdr*) which may exert negative influences on cell growth (Assoian & Zhu 1997). Serum starvation is also known to induce a PKC-binding protein, *sdt*-related gene product that binds to PKC-kinase (*SRBC*). Although the action of SRBC on PKC has not been clearly elucidated, it is speculated that SRBC binding to PKC has a negative effect on growth stimulation by inhibiting activation of PKC by Ca^{2+} (Gustincich & Schneider 1993).

More interestingly, priming endothelial cells with a pulse of FBS was sufficient to increase the percentage of proliferating cells in serum-starved cultures back to control serum levels. Re-exposure to serum may also induce both the down-regulation of *sdr* expression (Assoian & Zhu 1997) and re-entry of serum-starved quiescent cells from G_0 into G_1 towards the restriction point where cells are committed to continue to S phase (Durham *et al.* 1982). In addition to stimulation by different factors released from injured cells (McNeil *et al.* 1989, Malam-Souley *et al.* 1996, Sammak *et al.* 1997), serum primed cells can respond to the release from contact inhibition, which creates free space and allows for changes in cell shape. A postwound pulse of serum was insufficient for proliferation, suggesting serum derived signals at this time were not sufficient for endothelial cell proliferation. Therefore, a

prewound pulse of serum was a sufficient signal to prime quiescent cells for the induction of proliferation hours after mechanical wounding.

In addition, when the $[Ca^{2+}]_i$ rise was reduced by blocking Ca^{2+} influx, the percentage of proliferating cells was also reduced. Therefore, Ca^{2+} influx was specifically required during the time of wounding. This may be due to the fact that a calcium spike is able to stimulate cell progression past the commitment point of entry into S phase after serum stimulation allows cells in G_0 to re-enter the cell cycle (Takuwa *et al.* 1995). Although Ca^{2+} was necessary for wound-induced proliferation, it was not sufficient. Wounding serum-starved cells in high Ca^{2+} buffer augmented the increase in $[Ca^{2+}]_i$, but did not increase proliferation to serum control levels. Buffer containing 2.5 mM Ca^{2+} was used since this concentration was determined to produce maximal augmentation of the wound-induced rise in $[Ca^{2+}]_i$ without toxicity to the cells.

Finally, when serum-starved cells were first primed with serum then wounded in high Ca^{2+} buffer, serum and Ca^{2+} were at least additive in inducing proliferation to levels comparable to cells that had never been serum starved. Therefore, the sequential activation of cells by a priming pulse of serum followed by a transient rise in $[Ca^{2+}]_i$ induced by mechanical removal of cells from contact inhibition was necessary for normal proliferation in the area around the denuded zone. The Ca^{2+} transient was also required at the time of wounding and could only be delayed for a few minutes. Therefore, the signals, serum, release from contact inhibition and Ca^{2+}, must all take place in a defined sequence and were ineffective if temporally shifted or reordered.

The baseline level of proliferation seen in non-wounded cultures was probably induced by a small percentage of the cell population dying off and detaching from the substratum as the media was depleted (Todaro *et al.* 1965). This base line level of proliferation and subsequent increases in proliferation levels induced by mechanical wounding and other stimuli differ between normal and tumour cells. Tumour cells are less stringently regulated by the rules of

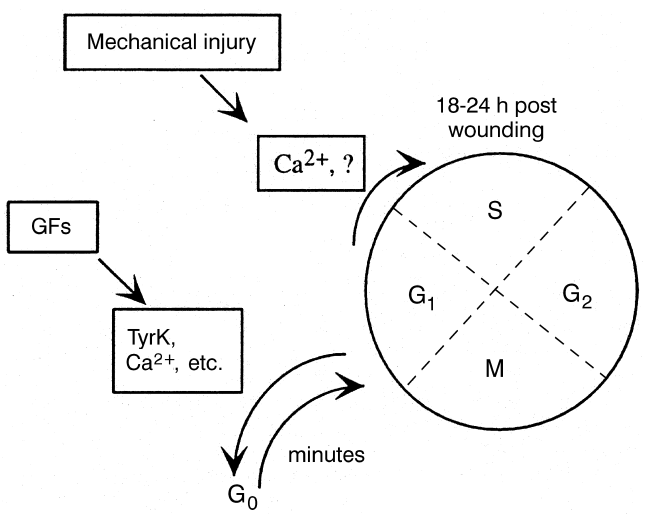


Figure 8. Model: Induction of proliferation 18–24 h post wounding. Serum priming induces quiescent serum-starved cells to re-enter the G_1 phase of the cell cycle from G_0 through induction of different pathways including Ca^{2+} and tyrosine kinases. Upon mechanical wounding, a transient $[Ca^{2+}]_i$ increase is induced and cells are released from contact inhibition, allowing for subsequent entry into the S phase of the cell cycle 18–24 h after wounding.

contact inhibition and do not express growth regulatory genes like *sdr* (Assoian & Zhu 1997). Interestingly, preliminary experiments indicate that other cellular activity induced upon mechanical wounding of tumour cells, like cell motility (P. O. T. Tran unpublished data, Kohn *et al.* 1995) and growth (Todaro *et al.* 1965, Whitfield *et al.* 1995) are also not regulated by Ca^{2+}. In addition, tumour cells may possess the ability to bypass the Ca^{2+} dependent points of cell cycle regulation by changes in morphology and transport systems (Whitfield *et al.* 1995, Izumi *et al.* 1997). Understanding these differences in the regulation of proliferation in normal cellular processes like re-endothelializaton and abnormal processes like tumour cell growth may lead to novel treatments to promote healing and prevent tumour progression.

Together, this study and other investigations suggest that a model for the induction of cellular proliferation in endothelial cell monolayers upon mechanical wounding (Figure 8). First, serum-starved quiescent cells need to be stimulated to return from the G_0 to the G_1 phase of the cell cycle before they can be induced to proliferate. The addition of serum provides the necessary signals which transcriptionally or translationally activate macromolecules important for the G_0/G_1 transition. This mechanism is believed to involve down-regulation of growth arrest genes like *sdr* (Gustincich *et al.* 1993) and activation of G_1-specific macromolecules possibly through a transient Ca^{2+} spike induced upon serum addition (Berridge 1995). Second, removal of contact inhibition by mechanical wounding not only provides free space for cell movement and growth, but also releases factors which stimulate a transient increase in $[Ca^{2+}]_i$. This Ca^{2+} signal may regulate the induction of cellular proliferation hours after the initial stimulus through its transcriptional activation of immediate early and late genes important for DNA synthesis like the AP-1 transcription factors and the cyclins, respectively.

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