A REDOX-SIGNALING MECHANISM FOR DENSITY-DEPENDENT INHIBITION OF CELL GROWTH

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Running Title: Impaired redox-signaling mediates contact inhibition.
SUMMARY

Reactive Oxygen Species (ROS) have recently drawn significant attention as putative mitogenic mediators downstream of activated Growth Factor receptors and oncogenic Ras; however, the possibility that redox-related mechanism also operate in the negative control of cell proliferation by inhibitory signals has not been investigated so far. Here we show that the arrest of growth induced by cell confluence (“contact inhibition”) is at least in part due to a decrease in the steady state levels of intracellular ROS, and consequent impairment of mitogenic redox signaling.

In confluent fibroblast cultures the decrease in the concentration of oxygen species was associated to diminished activity of the small GTPase Rac-1, a signal transducer directly involved in the ligand dependent generation of oxygen-derived molecules, and was effectively mimicked by exposure of sparse cultures to Dithiotreitol (DTT) and inhibitors of enzymes (Phospholipase A2 and Lipoxygenase) acting in the arachidonic acid cascade downstream of Growth factor receptors and Rac-1.

Sparse fibroblasts treated with non-toxic amounts of DTT underwent growth arrest, while low concentration of Hydrogen Peroxide significantly increased thymidine incorporation in confluent cultures, demonstrating a causal link between redox changes and growth control by cell density.

Removal of oxygen species from sparse cultures was accompanied by a drastic decrease of protein tyrosine phosphorylation following EGF stimulation, which reproduced, at a biochemical level, the signaling hallmarks of contact inhibition. Moreover, the cytosolic tyrosine phosphatase SHP-2 was identified as a putative target for redox signaling by cell density, since the enzyme itself and the associated substrates appear markedly dephosphorylated in both confluent and reductant-treated cells following exposure to EGF, and SHP-2 enzymatic activity is strongly activated by DTT in vitro. Taken together these data support a model in which impaired generation of ROS and increased PTPase activity impede mitogenic signaling in contact-inhibited cells.

Abbreviations used: 4-BPB; 4-Bromophenacyl-Bromide. DCF-DA; Dichlorofluorescein-diacetate. DMEM; Dulbecco’s Modified Eagle’s Medium. DMSO; Dimethylsulfoxide. DPI; Diphenileneiodonium. DTT; Dithiotreitol. ECL; Enhanced Chemo-Luminescence. EGF; Epidermal Growth Factor. FCS; Fetal Calf Serum. GST; Glutathione-S-Transferase. HBSS; Hank’s Balanced Salt Solution. HRP; Horseradish Peroxidase. JNK-1; Jun NH2-terminal Kinase 1. NDGA; Nordihydroguaiaretic Acid. PLA2; Phospholipase A2. p-NPP; p-Nitrophenil Phosphate. PDGF; Platelet Derived Growth Factor. PMSF; Phenylmethylsulfonyl Fluoride. PTP1B; Protein Tyrosine Phosphatase 1B. PTPase; Protein Tyrosine Phosphatase. ROS; Reactive Oxygen Species.
INTRODUCTION.

Significant evidence points to a role for oxygen derived reactive species (ROS) as mitogens for mammalian cells; this possibility is suggested by the fact that exogenous oxidants can induce quiescent cells entry into cell cycle (1), and are able to elicit signal transduction events, as for instance protein tyrosine phosphorylation and early gene activation, reminiscent of cell stimulation by Growth Factors (2). More importantly, “traditional” proliferative signals, as those delivered by the activation of growth-factor receptors and G proteins of the Ras family, are accompanied by intracellular production of endogenous oxygen species, which are in turn necessary for downstream propagation of mitogenic signaling. In fact ROS, and Hydrogen Peroxide in particular, have been convincingly shown to operate as key signaling molecules in the cascades triggered by PDGF (3), EGF (4), cytokine and antigen receptors (5, 6), and to be required for proliferative response to oncogenic Ras (7).

Observations on redox regulation of growth-factor signaling have been of special interest for the understanding of the molecular mechanism underlying the mitogenic properties of oxidants, and their potential involvement in proliferative disorders such as cancer and atherosclerosis. Most growth factors activate, through their tyrosine kinase receptors, an intracellular cascade of events involving, as central components, the tyrosine phosphorylation of the receptor itself and of a number of other substrates, the phosphotyrosine-dependent recruitment to cell membrane of multiple signal transducers and, eventually, the delivery of mitogenic stimuli to the nucleus (8). Although with remarkable exceptions, tyrosine phosphatases exert a general inhibitory effect on such cascade, by attenuating the intensity of phosphorylation signals initiated by activated receptors, and accelerating their extinction (9), as clearly shown by the emergence of deregulated cell growth in association to genetic inactivation of some specific member of this class of enzymes (10).

Tyrosine phosphatases are especially sensitive to redox regulation, and are easily inactivated by oxidation of a critical cysteine residue located in the catalytic site (11); they represent therefore a potential target for both exogenous and endogenously derived oxygen species. In fact, tyrosine phosphatase PTP1B is rapidly and transiently inactivated by ROS following EGF-receptor triggering (12), and some reports exist on redox regulation of phosphatase activities in physiological conditions (13).

In multicellular organisms, normal cell growth is strictly regulated not only by the local availability of growth factors but also by a number of positive and negative co-stimuli delivered by the extracellular matrix and the neighbouring cells. A clear example of such complex network is
provided by the negative regulation of normal cell growth by cell-cell contact, a phenomenon also known as “contact inhibition”. Normal adherent cells as well as some immortalized cell lines exit cell cycle and stop proliferating when grown to confluence, even in the presence of optimal amounts of growth factors. This contact dependent control of cell proliferation is believed to act in vivo during tissue regeneration and wound healing, and is also conceivably involved in tissue patterning during embryonic development (14). Cell transformation by oncogenes results in loss of contact inhibition; transformed cells maintain the capacity to divide at very high density, and pile up in foci, instead of growing in monolayer. The transforming capacity of molecules such as the tyrosine kinase Src and the G-protein Ras, as well as studies comparing growth factor signaling at different cell densities, have been of help in starting to elucidate the molecular basis of growth control by cell-cell contact. For instance, confluent cells have been shown to have decreased levels of protein tyrosine phosphorylation, associated to an increase in PTPase activity (15). Cell treatment with tyrosine phosphatase inhibitors relieves contact inhibition and allows cell growth at high saturation density (16). Confluent cells are also refractory to the mitogenic effects of Growth Factors such as EGF and PDGF, and such hyporesponsiveness correlates with accelerated dephosphorylation of the corresponding tyrosine kinase receptors (17). In addition, molecules associated to cell-cell adherent junction, such as cadherins, catenins and the growth factor receptor substrate p120 are also poorly phosphorylated in confluent endothelial cell cultures, but strongly phosphorylated by v-Src (18). The idea that tyrosine phosphatases are directly involved in contact inhibition is therefore largely accepted, also in view of the crucial role of protein tyrosine phosphorylation in transducing signals delivered by growth factor receptor as well as by adhesion molecules; however, the modality of regulation of these enzymes by cell density remains still unclear.

In view of the reported sensitivity of tyrosine phosphorylation cascades and tyrosine phosphatases to oxidants and reductants (19), and of the emerging importance of oxygen radicals as physiological regulators of cell proliferation (20), we asked whether a redox mechanism could be involved in the growth inhibitory signals delivered by cell-cell contact. Experiments here presented provide evidence that growth inhibition by cell density operates, at least in part, through a redox regulation of growth factor signaling, and, by extension, that the concentration of intracellular ROS may represent an important level of integration for positive and negative signals regulating cell proliferation.
EXPERIMENTAL PROCEDURES

Cell lines and Reagents. Swiss 3T3 murine fibroblasts and MRC-5 human embryonic fibroblasts were obtained from the Istituto Zoo-profilattico di Brescia (Brescia, Italy). Human adult fibroblasts immortalized by SV40 (Gm701) were a gift of Dr. Michael Jacobson (University College, London, UK). All cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) additioned with 10% heat-inactivated fetal calf serum (Eurobio, Les Ulis, France). MRC-5 cells were used between passages 28 and 35.

The Escherichia Coli strain expressing the glutathione S-transferase-PAK-CRIB domain fusion protein was a kind gift of Dr. J. Collard (Amsterdam, The Netherlands). The fusion protein was purified from bacterial lysate, induced with β-D-thiogalactopyranoside (Advanced Biotechnology), with glutathione-conjugated Sepharose 4B (Pharmacia, Uppsala, Sweden), in accordance with manufacturer’s recommendations.

Antibodies used in the present studies are: anti-Rac-1 (Transduction laboratories), anti-Phosphotyrosine (clone 4G10 from Upstate Biotechnology), anti-SHP-2 (Santa Cruz Biotechnology), anti-actin (Santa Cruz Biotechnology) HRP-conjugated secondary reagents (anti-mouse IgG and anti-rabbit IgG from Pharmacia [Uppsala, Sweden] and Biorad, respectively).

Standard reagents for protein electrophoresis and western blotting were from Biorad and Sigma: Dimethyl Sulfoxide (DMSO), Diphenileneiodonium (DPI), 4-Bromophenacyl Bromide (4-BPB), Nordihydroguaiaretic acid (NDGA), Dithiotreitol (DTT), p-Nitrophenil phosphate (p-NPP, Sigma 104), Sodium Orthovanadate, Hydrogen Peroxide and Human Epidermal Growth Factor (EGF) were purchased from Sigma (St. Louis, MO, USA). Reagents for Enhanced Chemo-luminescence (ECL), Protein-G Sepharose 4B and Glutathione Sepharose 4B were from Pharmacia. Dichlorofluorescein-diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR, USA).

[3H]methyl-thymidine was from Amersham Radiochemicals and autoradiography films from Kodak.

Cell proliferation assays. For [3H]-thymidine incorporation assay fibroblasts were seeded in 96 well flat bottom microtiter plates (Corning) in 200 µls of complete DMEM, with or without stimulants/inhibitors, at the following densities; MRC-5 cells: 3X10^4 /well (Dense), 10^4 /well (Subconfluent), 3X10^3 /well (sparse). Gm701 cells: 10^5 /well (Dense), 3X10^4 /well (Subconfluent), 10^4 /well (sparse). The well area is approximately 0.3 cm². After 40 hours of incubation in 37
°C/5% CO₂ humidified incubator. [³H]-Thymidine was added at 1uCi/well for additional 8 hours. Cells were then trypsinized, resuspended in 100 uLs of PBS/well and harvested with a semi-automatic cell-harvester. [³H]-Thymidine incorporation was measured using an automated β liquid scintillation counter. Counts are expressed as c.p.m./1000 cells seeded.

For the growth curve of Swiss 3T3 cells with or without DTT, cells were seeded at 3X10⁴/well in a 24 well plate and duplicate cultures were counted with a haemocytometer every 2 days. Culture medium was regularly changed every 3 days and fresh DTT added where necessary. In some experiments medium containing DTT was replaced after 8 days with standard medium after two washes with PBS.

**Measurement of intracellular ROS.** 3T3 and Gm701 cells were plated in 24-well plate (Corning) at the density of 6X10⁵ (Dense) or 10⁵ (Sparse) cells/well (corresponding to 3X10⁵ and 5X10⁴ cells/cm², respectively) in standard culture medium. MRC-5 were seeded at 1.25X10⁵ (Dense) or 2X10⁴ (Sparse) cells/well in DMEM. 16 hours later medium was replaced and antioxidants or enzyme inhibitors were added for 1 hour, followed by 1 hour incubation with 5 µg/ml of the oxidant-sensitive fluorescent dye DCF-DA. Cell were then detached from the substrate by trypsinization and immediately analysed by flow cytometry using a COULTER-EPICS Flow Cytometer equipped with an Argon laser lamp (FL-1, emission 480 nm, band pass filter 530 nm).

For analysis of ROS generation in response to EGF, Gm701 cells (sparse and dense) were incubated overnight in 1% FCS, washed with HBSS and treated with 100 ng/ml human EGF in HBSS for 5, 20 or 60 minutes at 37 °C. DCF-DA (dissolved in ethanol) was then added at the concentration of 20 µg/ml for 5 minutes. After fluorescent labeling, cells were quickly trypsinized and immediately subjected to flow cytometry.

**Cell stimulation and lysis.** Equal numbers (usually 10⁶) of cells plated at high or low density were stimulated with 100 ng/ml EGF (5 minutes), Pervanadate (5 minutes), or 100 µM Vanadate (1 hour) in HBSS, or left untreated. Pervanadate was prepared by mixing 100 mM Hydrogen Peroxide with 30 mM Sodium Orthovanadate. After 5 minutes on ice, Pervanadate was added to cells at 1:100 dilution.

To terminate stimulation, adherent fibroblasts were washed once with PBS and lysed in 1% Triton lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl Ph 8, 2 mM EDTA, 1 mM PMSF, 1ug/ml of aprotinin, leupeptin and pepstatin, and 1 mM sodium orthovanadate) with the help of a
rubber scraper. After 30 minutes lysis on ice, cell debris was spun down (14000 rpm at 4°C for 20 minutes) and supernatant kept for SDS PAGE or immunoprecipitation studies.

**Determination of Rac-1 activity in sparse and dense cultures.** GTP-bound (active) Rac-1 was precipitated by protein lysates from sparse and dense cultures with a GST fusion protein containing the CRIB domain of the Rac-activated kinase PAK, according to Sander et al.(21) Briefly, 10⁶ Gm701 or 2.5X10⁵ MRC-5 cells cells were lysed as described above 16 hours after seeding at high or low density. Precleared lysates were incubated with 5-10 ug of PAK/CRIB-GST fusion protein absorbed on Glutathione-Sepharose beads (Pharmacia). After 3 hours of incubation at 4°C in constant agitation pellets were washed in lysis buffer and PAK-bound proteins resolved by SDS-PAGE. Immunoreactive Rac-1 precipitated by PAK-GST was then quantified by anti-Rac western blot analysis. Total cell lysates were also immunoblotted with anti-Rac to determine the expression level of Rac-1 in sparse and dense cells.

**Analysis of protein tyrosine phosphorylation.** Tyrosine phosphorylation on total cell lysates was assessed by anti-phosphotyrosine immunoblot with the mouse monoclonal antibody 4G10 (Upstate Biotechnology). Immunocomplexes were detected on nitrocellulose membrane by Horseradish peroxidase-conjugated anti mouse IgG antiserum, followed by Enhanced Chemo-luminescence (Pharmacia) and autoradiography. Immediately after protein electro-transfer to nitrocellulose, equal protein loading was verified and molecular weight marker (Life Biotechnology) protein bands evidenced by reversible Ponceau S staining.

To assess the level of tyrosine phosphorylation of SHP-2 and SHP-2 associated proteins, the enzyme was immunoprecipitated with an affinity purified rabbit anti-SHP2 antiserum (Santa Cruz Biotechnology) bound to Protein-G Sepharose beads. After extensive wash, the immunoprecipitates were boiled and proteins resolved by SDS-PAGE, transferred to nitrocellulose and subjected to anti-phosphotyrosine immunoblot. The same membrane was subsequently stripped and immunoblotted with the anti SHP-2 antiserum, to verify that equal amounts of immunoreactive enzyme was present in all samples.

**Assay for SHP-2 phosphatase activity.** Phosphatase activity of anti-SHP2 immunoprecipitates was determined as described before (22). Briefly, immunocomplexes were washed twice in phosphatase buffer ( 62 mM Hepes, pH 7, 6.25 mM EDTA ) and resuspended in 100 uL of the same buffer containing 10 mM of the artificial substrate p-Nitrophenyl Phosphate (p-NPP), with or without 3 mM DTT. Incubation was carried out for 30 minutes at 37 °C, and stopped with 4
volumes of NaOH 200 mM. Substrate dephosphorylation (yellow product) was quantified spectrophotometrically (Absorbance at 410 nM). Sham immunoprecipitations with preimmune rabbit IgG were performed as negative controls.
RESULTS

Reduced generation of intracellular ROS in contact-inhibited fibroblasts. Adherent cells grown in monolayers undergo cell growth arrest upon reaching confluence. This is in particular true for normal fibroblasts, which are used, in view of this property, for routine focus formation assays. Average DNA synthesis, evaluated by $[^{3}]$H-thymidine incorporation, was drastically reduced in human embryonic fibroblasts (MRC-5) as a function of increasing cell density (Fig. 1A, left panel). This was also the case for Swiss 3T3 murine immortalized fibroblasts (not shown) and for human adult fibroblast immortalized by SV40 (GM701; 1A, right panel). Although the latter cell line was still remarkably sensitive to density dependent growth inhibition, infection by SV40 allowed proliferation at higher saturation density in comparison to normal human fibroblasts (1A and data not shown).

In all the considered cell lines, steady state levels of intracellular Reactive Oxygen Species (ROS) were significantly lower in confluent cells than in sparse cultures, as assessed by cytofluorimetric analysis following exposure to the ROS-sensitive fluorescent probe Dichlorofluorescein diacetate (DCF-DA)(Fig. 1B). Special co-cultures in which sparse and dense cells shared the same medium were used, in some experiments, to rule out the possible effect of pH and pO$_2$ on the observed difference (not shown). Interestingly, fluorescence shift between confluent and sparse cultures was slightly less pronounced in GM701 cells, which grow at higher density than normal cells.

A transient increase in the intracellular concentration of Hydrogen Peroxide has been reported as an important signaling event upon human cells stimulation with Epidermal Growth Factor (4). Such oxidative burst reversibly inhibits PTP1B (12) and likely other tyrosine phosphatases thereby promoting the propagation of tyrosine phosphorylation signals.

In agreement with these previous observations, EGF raises intracellular ROS concentration in Gm701 sparse cells (fig.1C); this effect is markedly attenuated in the same cells plated at high density, and follows a different kinetic, with a rapid decline after 20 minutes of stimulation, suggesting that redox signaling by EGF is impaired by cell confluence. It should be noticed that the culture density did not affect the expression of the EGF receptor (not shown).

Rac-1 GTPase is negatively regulated by cell density. The small GTPase Rac-1 mediates shape changes following cell stimulation by growth factors (23), and is required for cell cycle progression (24) and for cell transformation by oncogenic Ras (25). This transducer has recently been involved...
in the ligand (26) and adhesion (27) dependent generation of reactive oxygen species, and therefore represents a key component of the redox signaling machinery activated by mitogenic stimuli.

Active (i.e. GTP-bound) Rac-1 binds to and activates a number of downstream effectors, including the serine threonine kinase PAK (28). We have used an assay developed by Sander et al. and based on co-precipitation of active Rac with a PAK-GST fusion protein (21), to evaluate the level of activity of Rac-1 in cells plated at different densities. In MRC-5 and GM701 cells Rac-1 activity was found significantly higher in sparse than in confluent cultures, while no or minimal changes were observed in the expression level of the protein, assessed by western blot analysis (fig. 2A). It thus appears that, even in the presence of optimal amounts of serum derived growth factors, the activity of Rac-1 is negatively regulated by cell density and, most likely, by cell-cell contact.

Rac-1 can regulate at least two different sources of oxygen radicals; one is represented by phagocytic NADPH oxidase or similar enzyme complexes expressed by non phagocytic cells (29). The other one is the conversion to prostaglandins and leukotriens of arachidonic acid, whose release is stimulated by growth factors in a Rac-dependent fashion (30). Since both rac-1 activity and intracellular ROS generation are depressed in confluent cells, it is conceivable that a Rac-dependent radical source is involved in redox changes induced by cell density. Pretreatment of sparse Gm701 cells with the NADPH oxidase inhibitor Diphenileneiodonium (DPI) had no effect on the steady state concentration of intracellular ROS in sparse cells, ruling out the possibility that an NADPH oxidase-like complex is the target for ROS regulation by cell density (2B, b). Conversely, both the Phospholipase A2 inhibitor 4BPB and the Lipoxygenase inhibitor NDGA, which act at different point of the cascade leading to leukotrienes biosynthesis, reduced the intracellular concentration of ROS nearly to the level observed in dense cells (2B, c and d), indicating that the oxidative metabolism of arachidonic acid could be involved in negative redox signaling by cell-cell contact.

**Density dependent effects of reductants and oxidants on fibroblast proliferation.**

In order to establish a causal link between redox changes induced by cell confluence and contact inhibition of cell growth, the intracellular ROS concentration of sparse 3T3 cells was artificially lowered with the cell permeant reducing agent dithiothreitol (DTT), to mimic dense cells. The same concentration of DTT (2mM) which reduced intracellular ROS of sparse cells to the level observed in dense cultures (fig. 3A) had a profound inhibitory effect on 3T3 cell growth (fig. 3B). This effect was not due to a loss of cell viability (not shown), and proliferative capacity was partially recovered
after removal of DTT (3B), as it is consistent with a reversible block in cell progression through the cell cycle.

Similarly, in MRC-5 and GM701 cells plated at low density the incorporation of $[3^H]$-thymidine, which reflects the fraction of proliferating cells, was significantly and in a dose-dependent fashion reduced by DTT (fig. 4A). As observed for 3T3 cells, also in these cell lines the drug had no or minimal effect on cell viability. Moreover, unlike cell confluence, DTT did not affect the level of activity of Rac-1 in sparse GM701 cells (fig. 4B), suggesting that the substance acts on a redox-sensitive pathway downstream of Rac, likely by directly scavenging oxygen species or by increasing the levels of reduced glutathione.

Low concentration (10 µM) of Hydrogen Peroxide had an opposite effect than DTT on cell growth, in that it stimulated the proliferation of both MRC-5 and Gm701 fibroblasts (fig. 4B). This effect was clearly dependent on cell density, since it was observed in confluent and subconfluent cultures, but not in sparse cells (fig. 4B), in which the ROS concentration is constitutively elevated (see above).

Impaired protein tyrosine phosphorylation in confluent fibroblast cultures.

Impaired growth factor signaling and increased tyrosine phosphatase activity have been described by several authors in contact-inhibited cells (15, 17). Moreover, protein tyrosine phosphorylation is a well-established target for signal transduction by Hydrogen Peroxide (19). As an initial step to evaluate the relationship between reduced ROS generation and defective mitogenic signaling in confluent fibroblasts, we exposed 3T3 cells to the phosphatase inhibitor Sodium Orthovanadate; this inhibitor acts, at least in part, through a redox mechanism and requires, for maximum activity, a pro-oxidant environment (31). 1 hour treatment with 100 µM Vanadate induced massive protein tyrosine phosphorylation in sparse 3T3 cultures, while having a modest effects on dense cells (fig. 5A, lanes 3 and 4). In confluent cultures the effect of Vanadate was fully restored by exogenous 1 mM Hydrogen Peroxide (figure 5A, lane 5), suggesting that in dense cells a defect in oxygen species prevents phosphatase inhibition by Vanadate. Although Vanadate is a non-physiological stimulus, these results are consistent with the observation that the generation of endogenous oxygen species is defective in confluent cells and suggest that this defect could have a significant impact on redox-signaling through protein tyrosine phosphorylation.

In agreement with previous reports (17), accumulation of tyrosine phosphorylated proteins in Gm701 cells stimulated with Epidermal Growth Factor was also reduced by cell density (fig. 5B), in
a fashion which was not dependent on the total amount of proteins (fig 5B) nor on the expression level of the EGF receptor (not shown). As previously suggested (17), this difference was conceivably due to increased tyrosine phosphatase activity in dense cells; in fact, complete inhibition of PTPases by Pervanadate (Vanadate+Hydrogen Peroxide) induced comparable levels of phosphoproteins accumulation in sparse and dense cultures (fig. 5A, lanes 7 and 8), demonstrating that tyrosine kinase activities are not affected per se by cell density. Exposure of sparse cultures to DTT for 1 hour, a treatment which drastically reduced the level of intracellular oxygen species, almost completely prevented tyrosine phosphorylation in response to EGF (fig. 5A lanes 5 and 6), thereby mimicking the signaling defect observed in cells grown at high density (lanes 1 and 2).

**Modulation of the cytosolic phosphatase SHP-2 by cell density.**

As many other tyrosine phosphatases (11), the cytosolic protein tyrosine phosphatase SHP-2 is remarkably sensitive to redox regulation, and is rapidly inactivated by oxidants. In agreement with this concept, the reducing DTT significantly stimulated the activity of the cytosolic protein tyrosine phosphatase SHP-2 *in vitro*, suggesting that a reducing environment, as it is found in confluent cells, can promote protein dephosphorylation through the direct activation of PTPases (Fig. 6C). In EGF-stimulated cells, SHP-2 co-precipitates with the activated EGF receptor and a prominent 120 kD protein band, both proteins representing SHP-2 substrates (32). SHP-2 is also tyrosine phosphorylated upon EGF stimulation, and can rapidly auto-dephosphorylate (33). The level of phosphorylation of SHP-2 and SHP2 associated proteins can be therefore assumed as an indirect measure of SHP-2 activity *in vivo*. Phosphorylation of p180 (EGF-R) and p120 co-precipitated with SHP-2 was more pronounced in sparse Gm701 cells in comparison to dense cultures. This difference, which was also mirrored by the phosphorylation of SHP-2 in both Gm701 (fig. 6A) and MRC-5 (fig. 6B) cells, could be reproduced by exposure of sparse cells to the reducing agent DTT (fig. 6A, compare lanes 4 and 6). Pretreatment of sparse and dense cells with the strong PTP inhibitor Pervanadate led to a dramatic increase in the phosphorylation of SHP-2 and its associated substrates, and abolished differences between the two cell populations (6A, lanes 7 and 8); this finding is consistent with the idea that differences in the phosphorylation of SHP-2 and SHP-2 associated proteins between sparse and dense cells depend mainly on the level of activity of the enzyme in the above culture conditions, and, by extension, that SHP-2 is functionally modulated by cell-cell contact.
DISCUSSION

Recent observations on the signaling properties of reactive oxygen species have opened a new perspective in the correlation between oxidative stress and cancer. Oxygen radicals in fact not only damage DNA, but also directly promote cell growth, and endogenously generated oxidants have been convincingly shown to participate in the signaling cascade initiated by growth factor receptor and active Ras (20).

Defects in growth inhibitory signals contribute to the deregulated cell proliferation during carcinogenesis. For instance, loss of contact inhibition is a typical feature of transformed cells, and reduced responsiveness to TGF-beta signals is commonly found in some classes of neoplasms, like pancreatic cancers (34). Although of crucial importance for the molecular understanding of cell progression to cancer, the biochemical mechanisms underlying the intracellular delivery of anti-proliferative signals are still poorly understood in comparison to the well-established signaling cascades leading to mitogenesis. In the present work we provide evidence for a novel mechanism of negative control of cell growth by cell-cell contact, consisting in the reduced production of endogenous ROS and in impaired redox-signaling by growth factor receptors.

Unlike many studies conducted on cells grown to confluence over several days of culture, in our experimental model cells are directly plated at low or high (saturation) densities, and biochemical parameters (including ROS generation) are evaluated after not more than 16 hours later, when cell distribution along the cell cycle has not yet significantly changed. This procedure, as already suggested by other authors (35), should allow the detection of early density-related signaling events which are unlikely to represent secondary consequences of cell cycle arrest.

Of central importance, among the presented data is the observation that steady state as well as EGF-induced levels of intracellular oxygen radicals are significantly reduced in fibroblasts plated at high density, in comparison to sparse cultures. Changes in the levels of intracellular ROS can be already detected 16 hours after cell plating, and are function of the surface area, but not of the volume of medium in which cells are resuspended (not shown). This observation, together with the fact that redox properties of dense/sparse cells cannot be transmitted by the corresponding cell supernatants (not shown), points to a direct involvement of cell-cell contacts in the modification of intracellular redox environment in confluent cultures. It should be noted that a number of controls were performed in order to rule out potential experimental artifacts due, for instance, to reduced oxygen concentration in dense cultures, medium acidification or reduced permeability of dense cells to the fluorescent probe DCF-DA (data not shown).
The finding of reduced Rac-1 activity in cells plated at high density may provide the biochemical link between cell-cell contact and impaired redox signaling in confluent monolayers. Rac-1 is in fact necessary for the generation of the oxygen species involved in mitogenic signaling by Growth factor receptors and oncogenic Ras (20). Moreover, Rac-1 activity is required for cell cycle progression through the G2/S boundary, as demonstrated by transfection of dominant negative forms of this GTPases in rodent fibroblasts (24). More important, active forms of Rac-1 induce focus formation, and selectively increase the cell saturation density, while having little effect on exponential fibroblast growth (25). Therefore, independent of its involvement in signal transduction by oxygen species, the observation that Rac-1, an important effector of Ras function, is negatively regulated by cell density is relevant for the understanding of negative signaling in contact-inhibited cells. Among G proteins, such density-dependence has been so far reported only for the small GTPase Rap-1 (36), while Ras has been found to be unaffected by cell confluence (37), and is unlikely to be involved in contact inhibition. Finally, our findings are in line with the recent observation that cell density inhibits the activity of the stress kinases JNK-1 and p38, two well established targets for both Rac-1 and oxidant signaling (37).

Rac-1 inhibition by cell confluence could be mediated by integrin or surface receptor (such as cadherins (38) or contactinhibin (39)) signaling; alternatively, this transducer could be mechanically regulated by cell-cell contact through spatial constraint or changes in the cell shape, both conditions which impede cell proliferation (40, 41). Additional studies will be necessary to clarify this important point.

While in many cellular models, such as chondrocytes (5), vascular smooth muscle cells (3) and T lymphocytes (6), flavin oxido-reductases sensitive to diphenileneiodonium account for ROS production in response to receptor ligands, the present data suggest that enzymes involved in the oxidative metabolism of arachidonate, and lipoxygenases in particular, represent the potential sources of oxygen radicals negatively modulated by cell-cell contact. This observation is consistent with the fact that lipoxygenases are a major source of ROS in growing fibroblasts (42), and that their activity is stimulated by growth factors in a Rac-1 dependent fashion (30).

Products of arachidonate metabolism have recently received significant attention as endogenous promoters of carcinogenesis. Cyclooxygenase 2, for instance, is overexpressed in an high percentage of colon carcinomas, and its pharmacological inhibition prevents experimental cancer (43). A soluble form of phospholipase A2, on the other hand, has been identified as a major factor of susceptibility to cancer in genetically predisposed mice (44). Finally, inhibitors of lipoxygenases have been reported to inhibit cell growth in vitro (45). While these observations have drawn
attention mainly on the lipid products derived from arachidonic acid, it is conceivable that oxygen radicals generated as by-product of prostaglandins and leukotriens biosynthesis may play a role in stimulating cell proliferation and tumor promotion. In view of these concepts, the idea that arachidonic metabolism could be inhibited by cell-cell contact through Rac-1, although only indirectly supported by our data on intracellular ROS measurement in cells treated with 4BPB and NDGA, is intriguing and currently under further investigation. When evaluating this possibility, however, it should be considered that a direct demonstration that redox changes in dense cultures are consequence of Rac-1 inhibition has not been provided in the present study; transfection experiments with constitutively active and dominant negative forms of the GTPase, currently in process, will help to conclusively address the causal link between Rac-1 downregulation and impaired redox signaling in dense cells.

A critical point of the presented work is the demonstration that cell density controls cell growth, at least in part, through the intracellular concentration of oxygen species. This novel idea is supported by the growth inhibitory effect of the reducing agent DTT on sparse cells (figures 3B and 4A) and, even more important, by the partial escape from contact inhibition of confluent cells exposed to exogenous Hydrogen Peroxide (fig. 5B). Although relief of density-dependent growth arrest in those cells is not complete, it is relevant that H2O2 is mitogenic only on dense cells (where intracellular ROS concentration has conceivably dropped below a threshold-level compatible with cell cycle progression) whereas it has no effect on sparse cultures. This observation further confirms that redox signaling is significantly altered by cell density, and provides a new biochemical mechanism for deregulation of cell growth by oxidant species.

While the intracellular molecules which mediate the effects of redox changes on cell proliferation have not been identified in the present study, our data point to protein tyrosine phosphorylation/dephosphorylation as a key mechanism of redox signaling by cell density. The finding that some signaling characteristics of confluent cells, such as impaired tyrosine phosphorylation and increased tyrosine phosphatase activity, can be reproduced in sparse cells by the reducing agent DTT, further strengthen the causal link between lowered concentration of oxygen radicals and defective responsiveness to growth factors in contact-inhibition.

The extracellular domain of many growth factor receptors, including the EGF receptor, contains disulfide bonds potentially sensitive to reduction by DTT, with consequent impaired signal transduction. Since this possibility has not directly ruled out in the present study, the inhibitory effect of DTT on protein tyrosine phosphorylation should be interpreted with caution. However, it should be noted that DTT at the concentrations here used in not or minimally toxic, and seems to
act at a level downstream of Rac-1 activation (and therefore of receptor binding to growth factors) as indicated by the normal Rac-1 activity in DTT treated cells (fig. 4C and 7). Moreover, the lipoxygenase inhibitor NDGA has a similar inhibitory effect as DTT on EGF induced protein phosphorylation, while lacking thiol reducing properties (data not shown).

The activity of the tyrosine phosphatase SHP-2 is significantly stimulated by reducing conditions in vitro (fig. 6C), and indirect evidence has been provided that this phosphatase is more active in confluent cells than in sparse cultures (6A and B). These observation, in view of the well established role for this enzyme as an intracellular transducer downstream of growth factor receptors and integrins (9), qualify the tyrosine phosphatase SHP2 as a molecular model for oxidative regulation of cell growth, and as a putative target for redox-signaling by cell density. Further experiments will help to directly assess the relative role of this enzyme in density-dependent cell cycle arrest.

In conclusion, the data here presented are consistent with a model in which defective redox signaling through Rac-1 impairs mitogenesis by growth factors and mediates, at least in part, contact inhibition (fig. 7). While additional studies will be necessary to understand in details the molecular mechanism through which redox changes observed in confluent cells are induced and translated into cell cycle regulatory events, the present work outlines a novel, ROS-dependent circuit of regulation of cell growth, which may help to better understand the role of oxidative stress in proliferative disorders such as atherosclerosis and cancer.

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FIGURE LEGENDS

Figure 1. Reduced concentration of intracellular ROS in fibroblasts undergoing density-dependent growth arrest.

A. [³H]-Thymidine incorporation in primary and immortalized fibroblasts seeded at different densities. Cells were incubated for 40 hours + 8 hours pulse with 1 µCi/ well [³H]-Thymidine. Values are calculated as (Total c.p.m./number of cells seeded at time 0) X1000 and represent mean ± SD of triplicate cultures. In both cell lines DNA synthesis is significantly inhibited by cell confluence.

B. Flow cytometry analysis of cells loaded with the redox-sensitive fluorescent probe DCF-DA. Cells were labeled with 5 µg/ml DCF-DA for 30 minutes after 16 hours incubation at high or low plating density (see Experimental Procedures), detached from the substrate and analyzed by flow cytometry (green fluorescence, emission 488 nm, excitation 530 nm). Filled histograms: sparse cells. Empty histograms: dense cells.

C. Confluent Gm701 cells fail to increase intracellular ROS in response to EGF. Intracellular redox changes at different times after EGF (100 ng/ml) stimulation were monitored with a short (5 minutes) pulse of 20 µg/ml DCF-DA followed by flow cyrometry. Values are calculated as fluorescence variation, i.e. as (Mean Cell Fluorescence of treated samples - Mean Cell Fluorescence of unstimulated control), and are mean±SD of duplicate samples. Relative fluorescence increase was about 50% in sparse cells.

All experiments were repeated several times with reproducible results.

Figure 2. Impaired Rac-1 signaling and ROS generation by arachidonate methabolism in confluent fibroblasts.

A. The activity of Rac-1, an upstream regulator of leukotrienes biosynthesis in response to growth factors, was measured in sparse and dense cells using the pull-down assay developed by Sander et al. (21). The amount of GTP-bound Rac-1 is significantly reduced in cells plated at high density (upper panels) while no or minimal changes are observed in the amount of total Rac-1 (lower panels). Representative of at least two independent experiments.

B. Inhibitors of the leukotriene biosynthesis pathway, but not the NADPH oxidase inhibitor DPI, decrease the concentration of intracellular ROS in sparsely growing fibroblasts. 4BPB
(phospholipase A2 inhibitor, 20 µM), NDGA (5-lipoxygenase inhibitor, 5 µM), DPI (flavin oxidase inhibitor, 10 µM) and DMSO (vehicle control, 1:500) were added 1 hour before fluorescent labelling with DCF-DA. Numbers beside histograms indicate Mean cell fluorescence. Fluorescence of dense cells was not significantly affected by the indicated treatments.

Figure 3. Cell-permeant reducing agent Dithiotreitol arrests the growth of Swiss 3T3 cells.
A. DTT (2mM) significantly reduces the level of intracellular ROS in sparse 3T3 cells, thereby mimicking the effect of cell confluence. Intracellular ROS after 1 hour exposure to 2mM DTT were measured as in figures 1 and 2. Filled histogram: sparse cells; empty histogram with black line: dense cells; empty histogram with gray line: sparse cells + DTT
B. Reversible effect of DTT on Swiss 3T3 cell growth. 3x10⁴ cells were seeded in a 24 well plate in standard DMEM or DMEM containing 2mM DTT. Cell were counted every 2 days with an haemocytometer. Medium was replaced every 3 days. Numbers are Mean ± SD of duplicate wells. Figure representative of two independent experiments.

Figure 4. Effects of DTT and Hydrogen Peroxide on fibroblast proliferation.
A. (Upper Panel) Dose-dependent antiproliferative effect of DTT on MRC-5 and Gm701 cells. Sparse cultures (3X10³ cells/well for MRC-5 and 10⁴ cells/well for Gm701) were incubated for 48 hours in the presence of the indicated concentration of DTT. During the last 8 hours cells were labeled with 1mCi/well of [³H]-Thymidine. Values are expressed as percentage of radioactivity incorporation with respect to control cultures, and represent Mean±SD of triplicate wells.
(Lower panel) DTT lowers the concentration of intracellular ROS without affecting Rac-1 activity. Sparse (S), dense (D) and DTT- treated (S+DTT) Gm701 cells were subjected to the pull down assay for Rac-1 activity as described in in figure 2. The bands corresponding to GTP-bound (active) and total Rac-1 are indicated by arrows.
B. Hydrogen Peroxide increases proliferation of confluent cells. Cell proliferation at different densities was measured as in figure 1A, in the presence (white columns) or absence (black columns) of 10 µM Hydrogen Peroxide. Values are expressed as percentage of untreated sample (black columns) for each density. H₂O₂ is mitogenic in dense or suclonfluent cultures, but not for sparse cells. Numbers are Mean±SD of triplicate samples.

Figure 5. Redox modulation of protein tyrosine phosphorylation by cell density.
A. Effect of cell density on protein tyrosine phosphorylation induced by the phosphatase inhibitor Sodium Orthovanadate in 3T3 cells. Dense (D) and Sparse (S) cells were exposed to 100 µM Vanadate in HBSS or to HBSS alone for 1 hour. In lane 5, 1 mM Hydrogen Peroxide was added for the last 5 minutes of incubation. After cell lysis, total protein extracts (about 25 µg) were electrophoresed and immunoblotted with antiphosphotyrosine antibody 4G10. Figure representative of two independent experiments.

B. Reducing conditions created by either cell confluence or DTT prevent protein tyrosine phosphorylation in response to EGF. Gm701 cells were plated at high or low density 16 hours before stimulation with EGF (100 ng/ml in HBSS for 5 minutes), Pervanadate (1:100 for 5 minutes) or HBSS alone. DTT was added for 1 hour and removed before stimulation. Total protein lysates were analyzed for protein tyrosine phosphorylation (upper panel) or actin expression (loading control, lower panel) as described in Experimental Procedures. Figure representative of several independent experiments.

Figure 6. Identification of SHP-2 as a tyrosine phosphatase modulated by cell density.

A. (Upper panel) Tyrosine phosphorylation analysis of SHP-2 and its associated substrates in sparse and dense cultures. Gm701 cells were treated as in figure 5B, except that EGF was given for 20 minutes before cell lysis (lanes 2, 4 and 6). Total lysates were immunoprecipitated with anti SHP-2 antibody and immunocomplexes subjected to antiphosphotyrosine immunoblot. Phosphoproteins corresponding to the EGF receptor and p120, as well as a weak band of 70 kD, visible in lane 4 and corresponding to SHP-2, are indicated by arrows. Differences in protein tyrosine phosphorylation were independent from the amount of immunoprecipitated SHP-2 (lower panel). A slight mobility retardation of the SHP-2 band, likely due to heavy tyrosine phosphorylation, was consistently observed in samples treated with Pervanadate (lower panel, lanes 7 and 8).

B. EGF-induced tyrosine phosphorylation of SHP-2 in MRC-5 cells is modulated by cell density. Dense and sparse MRC-5 fibroblasts were stimulated with EGF for 20 minutes. Cell lysates (about 1 mg proteins) were immunoprecipitated with anti SHP-2 antibody, and immunocomplexes analysed by anti phosphotyrosine immunoblot. The phospho SHP-2 band, visible only in lane 3, is indicated by arrow. Immunoreactive SHP-2 was equally present in all lanes (lower panel, arrow).

C. In vitro Activity of SHP-2 is enhanced by a reducing environment. SHP-2 was immunoprecipitated by Gm701 cell lysate and assayed for phosphatase activity towards the artificial substrate p-NPP in vitro. DTT was added at a concentration (3mM) which, in intact cells, reproduces redox changes induced by cell confluence. Incubation was carried out for 30 minutes at
37 °C, and stopped with four volumes of 200 mM NaOH. Development of yellow colour was quantified spectrophotometrically (Absorbance at 410 nM). Activity of sham immunoprecipitates was also measured as negative control (Preimm.). Values are Mean±SD of duplicate samples. Figure representative of two independent experiments.

Figure 7. A model for inhibitory redox signaling by cell density.

In the present model impaired generation of Reactive Oxygen Species plays a central role in growth inhibition by cell density. This defect (maybe due to changes in cell shape or inhibitory integrin signaling) results in an intracellular environment unfavorable to protein tyrosine phosphorylation and eventually in cell growth arrest. See also Discussion.
REFERENCES


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A

MRC-5   GM701

GST-PAK pull-down

Total lysate

Blot: anti Rac-1

B

a

B

b

C

D

DCF-DA fluorescence

Ctrl

2.8

5.3

DPI

5.3

4BPPB

2.7

NDGA

3.2
Cell confluence

- Mechanical constraint
- Cell shape change
- Integrin signaling

↓ ROS
Impaired redox signaling

Increased tyrosine phosphatase activity

Growth arrest
A redox-signaling mechanism for density-dependent inhibition of cell growth
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