Contact-induced growth inhibition is a characteristic feature of normal cells grown in monolayer. The importance of reversible tyrosine phosphorylation in mitogenic signaling, together with earlier reports of increased levels of protein-tyrosine phosphatases (PTPs) in densely cultured cells, has led to the proposal that PTPs may be involved in mediating contact inhibition of cell growth. We have compared net levels of ligand-induced tyrosine phosphorylation of the epidermal growth factor (EGF) receptor in mink lung epithelial cells cultured under sparse or dense conditions. The levels of net tyrosine phosphorylation of the stimulated EGF receptor was found to be more than 4-fold higher in sparse cultures. This difference was greatly reduced when cells were pretreated with the PTP inhibitor phenylarsine oxide. Monitoring of dephosphorylation rates in vivo of the stimulated EGF receptors revealed increased EGF receptor-directed PTP activity in dense cultures. The platelet-derived growth factor β-receptor, expressed in stably transfected porcine aortic endothelial cells, also displayed lower levels of ligand induced net tyrosine phosphorylation in cells from dense cultures. This density-dependent difference in tyrosine phosphorylation was reduced by pretreatment of cultures with the PTP inhibitor orthovanadate. A PTP-mediated decrease of the in vivo net levels of ligand-induced tyrosine phosphorylation of EGF and platelet-derived growth factor receptors in cells at high density have thus been demonstrated. Loss of this previously unnoticed regulatory pathway may be involved in cellular transformation.

Support for this hypothesis (2). During recent years additional data have been obtained that support this idea. Increased PTP activity in membrane fractions or cell lysates from cells harvested at high cell densities has been reported (3–5). Cell density-dependent up-regulation of the receptor-like PTPs density-enhanced phosphatase-1 and PTP-μ at the protein level has been demonstrated (6, 7). Comparison of mRNA levels of PTPs in growing and contact-inhibited cells also revealed higher mRNA levels for PTP-μ and PTP-λ in dense cells (8). The demonstration of specific homophilic interactions of the extraacellular domains of the transmembrane PTP-μ and A5 also points toward a role in cell contact-induced signaling (9–11). Finally, the recent demonstration of physical association between PTP-μ and cadherins is compatible with a regulatory role for PTP-μ in signaling triggered by cell contact (12).

The ability of PTPs to reduce receptor tyrosine phosphorylation in vivo has been directly demonstrated in co-transfection experiments of transiently expressed tyrosine kinase receptors and PTPs (13). In addition, signaling through the PDGF receptor was shown to be attenuated in cells stably expressing transfected CD45 (14, 15). Also, antisense mediated blocking of endogenous leukocyte antigen-related phosphatase increased ligand-induced tyrosine phosphorylation of the insulin-receptor (16). Together, these results demonstrate that the net level of tyrosine phosphorylation of tyrosine kinase receptors in vivo will depend on the combined action of tyrosine kinases themselves and counteracting PTPs.

In this study we have compared net levels of growth factor-induced receptor tyrosine phosphorylation in cells cultured under sparse or dense conditions. A density-dependent reduction in ligand-induced tyrosine phosphorylation of EGF receptors in mink lung epithelial cells, as well as of transfected PDGF β-receptors in endothelial cells, is demonstrated. In both cases the density-dependent decrease in growth factor receptor tyrosine phosphorylation was reduced if cells were pretreated with PTP inhibitors. Also, monitoring of the in vivo dephosphorylation of the EGF receptor revealed an increased rate of dephosphorylation in cells from dense cultures.

**MATERIALS AND METHODS**

**Cell Culture—Mink lung epithelial cells, Mv1Lu (ATCC), and PDGF β-receptor expressing porcine aortic endothelial cells (PDGF-βR-PAE-cells) (17)** were grown in Dulbecco's modified Eagle's medium and Ham's F-12 medium, respectively, containing 10% fetal bovine serum and supplemented with antibiotics. In the experiments, Mv1Lu cells were seeded to give a density of less than 18,000 cells/cm² (sparse cultures) and more than 130,000 cells/cm² (dense cultures) at the time of the experiments. PDGF-βR-PAE-cells were used at densities of less than 40,000 cells/cm² (sparse cultures) and more than 150,000 cells/cm² (dense cultures).

**[3H]Thymidine Incorporation—** Cells were plated in 6-well dishes. After adherence, cells were serum-starved for 48 h. Cells were then stimulated in triplicate with different concentrations of growth factors. At the time of stimulation, cells in separate wells were counted. For
stimulation with EGF (Boehringer Mannheim) cells were incubated with EGF for 16 h, and incubation continued for 4 h in the presence of \(^{3}H\)thymidine (0.5 \(\mu\)Ci/ml). When PDGF-BB was used for stimulation, ligand and \(^{3}H\)thymidine were added simultaneously and incubation lasted for 24 h. High molecular mass \(^{3}H\)radioactivity was precipitated by incubating the cells with 5% trichloroacetic acid for 20 min on ice. After two washes with cold water the precipitates were solubilized in 1 m NaOH and acidified using 2 m HCl. Incorporation of \(^{3}H\)radioactivity was determined by liquid scintillation counting.

Receptor Binding Assay—EGF was \(^{125}\)I-labeled with the chloramin-T method to a specific activity of 75,000 cpm/ng. Sparse and dense cultures seeded in 6-well dishes were incubated for 2 h on ice with 2 ng/ml of \(^{125}\)I-EGF only or together with various concentrations of unlabeled EGF. Unspecific binding was determined by adding 300-fold excess of unlabeled EGF. After washing the cells and lysing them in 1 ml of 1% Triton X-100, 10% glycerol, and 20 mm Tris, pH 7.5, cell-associated radioactivity was measured in a \(\gamma\) -counter. The data were then subjected to Scatchard analysis. The cell number was determined by counting cells from parallel cultures.

Immunoblotting of Receptor Tyrosine Phosphorylation—In a typical experiment, 5 \(\times\) \(10^6\) cells were used for each precipitation. Cell numbers in the sparse and dense cultures were determined by counting cells plated at the same time as those used for the experiment. After overnight serum starvation cells were stimulated with 100 ng/ml growth factor for 3 h at 4°C and 5 min in 37°C. Prior to stimulation, cells treated with PTP inhibitors were incubated with 40 \(\mu\)M PAO at 37°C for 10 min or with 100 \(\mu\)M orthovanadate at 37°C for 1 h. After stimulation, cells were washed twice with phosphate-buffered saline and lysed in 1% Nonidet P-40, 0.15 m NaCl, 20 mm Tris, pH 7.5, 5 mm EDTA, 10% glycerol, 1 mm phenylmethylsulfonyl fluoride, 1\% Trasylol, 1 mm orthovanadate, and 100 \(\mu\)M PAO for 15 min at 4°C. After centrifugation, lysates were incubated with WGA-Sepharose (Pharmacia Biotech Inc.) for 3 h at 4°C. After three washes with lysis buffer, the precipitates were heated for 5 min at 95°C in SDS-sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes using semidyel electrophoresis. Membranes were blocked and probed with antiphosphotyrosine antibody PY20 (Transduction Laboratories, Affiniti Research Products, Ltd.), and after incubation with horseradish peroxidase-conjugated anti-mouse antibodies, tyrosine phosphorylated proteins were detected by enhanced chemiluminescence (ECL, Amersham Corp.). For EGF receptor detection, WGA-Sepharose fractions were analyzed by immunoblotting using EGF receptor antibodies (Affiniti Research Products, Ltd.).

In Vivo Dephosphorylation—Following stimulation, cells were transferred to ice, and the EGF receptor kinase blocker AG1517 (also PD153035) (18) in Me2SO was added to a final concentration of 1 \(\mu\)M. Only Me2SO was added in the controls. Dephosphorylation was stopped by lysis of the cells as described above. Collection of WGA-Sepharose fractions and immunoblotting were also made as described above.

RESULTS

Cells of Sparse and Dense Cultures of Mv1Lu Cells Differ in EGF Response but Express Equal Amounts of EGF Receptors—Mv1Lu are nontumorigenic mink lung epithelial cells. To demonstrate cell density-dependent growth inhibition of these cells, \(^{3}H\)thymidine incorporation assays were performed after EGF stimulation of serum-starved cells seeded under sparse or dense conditions (Fig. 1). Representative photomicrographs of cells at densities of 1.3 \(\times\) \(10^5\) cells/cm\(^2\) and 1.3 \(\times\) \(10^4\) cells/cm\(^2\), respectively, are shown in Fig. 1A. As shown in Fig. 1B, stimulation of sparse cells led to a dose-dependent EGF-stimulated increase in \(^{3}H\)thymidine uptake, with more than 4-fold maximum response compared with unstimulated cells. In contrast, stimulation of dense cells led only to a minor increase in DNA synthesis as compared with unstimulated dense cells. Calculated as \(^{3}H\)thymidine incorporation per cell, the difference between sparse and dense cells was more than 20-fold (Fig. 1B). Mv1Lu cells thus display a density-dependent EGF response typical of normal, nontransformed cells.

For further comparison of ligand-induced tyrosine phosphorylation of the EGF receptor in sparse and dense cultures, it was of importance to compare the number of EGF receptors on sparsely and densely cultured cells as well as the receptor affinities. To that end EGF binding experiments were performed with \(^{125}\)I-EGF, and results were subjected to Scatchard analysis. Fig. 1C shows the results from one such experiment. Both sparse and dense cells were found to express approximately 10,000 receptors/cell, and \(K_d\) values for the receptors in sparse and dense cells were determined to be 0.8 \(nm\) and 0.9 \(nm\), respectively. Thus, neither with regard to receptor number per cell nor \(K_d\) were any major differences observed between sparse and dense cells. That sparsely and densely cultured cells express equal amounts of EGF receptors was also confirmed by
immunoblot analysis. As shown in Fig.1D, no difference in the amounts of EGF receptor was found between cells harvested at sparse or dense culture conditions.

Sparse and Dense Mv1Lu Cultures Differ in EGF-induced Tyrosine Phosphorylation of the EGF Receptor—To compare ligand-induced EGF receptor tyrosine phosphorylation in cells at sparse or dense culture conditions, cells were allowed to bind EGF for 60 min at 4 °C and then transferred to 37 °C for 5 min. WGA fractions from lysates corresponding to equal number of cells from sparse and dense cultures was isolated, and parts of the material were then subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Levels of ligand-induced receptor tyrosine phosphorylation was then semiquantitatively determined by immunoblotting with phosphotyrosine antibodies. Dramatically lowered ligand-induced tyrosine phosphorylation of EGF receptors was observed in the dense cultures (Fig. 2). Because the signal in the lane corresponding to 80% of the lysates from the dense cultures is weaker than that from 20% of the lysates from sparse cultures, we conclude that the difference is at least 4-fold.

The Difference in EGF-induced Receptor Tyrosine Phosphorylation between Sparse and Dense Cultures Is Reduced by Pretreatment of Cells with PTP Inhibitors—Because the binding experiments had not revealed any differences in the binding of EGF to sparse and dense cultures, the difference observed in ligand-induced EGF receptor tyrosine phosphorylation reflects differences in the intrinsic receptor kinase activity or in the activity of PTPs acting at the EGF receptors. To investigate if the difference reflects increased PTP activity directed at the EGF receptor, cultures were preincubated in the presence or the absence of the PTP inhibitor PAO for 10 min (19). After ligand stimulation, receptor phosphorylation was analyzed as described above. As shown in Fig. 3, pretreatment of Mv1Lu cells with PAO dramatically reduced the density-dependent difference in ligand-induced receptor tyrosine phosphorylation. We therefore conclude that the observed difference in net tyrosine phosphorylation is caused by increased EGF receptor-directed PTP activity.

Tyrosine Phosphorylated EGF Receptors Are More Rapidly Dephosphorylated in Dense Cultures—To more directly demonstrate increased EGF receptor directed PTP activity in dense cultures, we applied a recently described method to monitor the in vivo dephosphorylation rate of tyrosine kinase receptors (20). In this method receptors are activated by ligand stimulation of intact cells; thereafter the kinase activity is blocked by the addition of rapidly acting kinase inhibitors belonging to the tyrphostin type. The rate of dephosphorylation can then be monitored by analyzing the amount of tyrosine phosphorylated receptors in cells harvested at various time points after addition of kinase inhibitor. This method was used to compare the rate of EGF receptor dephosphorylation in sparse and dense cultures (Fig. 4). After stimulation with EGF, cells were transferred to ice and exposed to either vehicle alone or the EGF receptor-specific tyrphostin AG1517. After indicated time points cells were lysed, and EGF receptor tyrosine phosphorylation was determined as above. In sparse cultures (Fig. 4, left panel) tyrosine phosphorylated receptors were easily demonstrated after 10 min of incubation with or without the kinase inhibitor, indicating very low receptor directed PTP activity in these cells. In contrast, in samples from dense cultures (Fig. 4, right panel), a decrease in the net levels of tyrosine phosphorylation of the EGF receptor is seen over time in dense cultures treated with vehicle only, indicating a net dephosphorylation under these conditions. Furthermore, this rate is dramatically increased in cells exposed to AG1517. Already after 5 min of incubation with kinase inhibitor, the levels of phosphotyrosine had decreased below the sensitivity of this assay. Thus, these experiments directly demonstrate increased EGF receptor-directed PTP activity in vivo in dense as compared with sparse cultures.

PTP-mediated Differences in PDGF-induced Tyrosine Phosphorylation of the PDGF β-Receptor between Sparse and Dense Cultures of PDGF-βR-PAE Cells—To investigate whether a cell density-dependent difference in growth factor receptor phosphorylation could be demonstrated also in other settings, we performed similar analysis of the PDGF β-receptor. The amounts of endogenous PDGF β-receptors in fibroblasts are transcriptionally regulated in a cell density-dependent manner (21). We therefore choose to study the transfected PDGF β-receptor in porcine aortic endothelial cells (PDGF-βR-PAE cells). These cells bind equal amounts of PDGF under sparse and dense culture conditions (data not shown). As shown in Fig. 5A, these cells show a density-dependent response to PDGF-BB in an [3H]thymidine incorporation assay. As with the EGF receptor in Mv1Lu cells, we could demonstrate a density-dependent difference in ligand-induced tyrosine phosphorylation of the PDGF β-receptor (Fig. 5B, left part). Also, this difference could be reduced by pretreatment of the cells with the PTP inhibitor orthovanadate (Fig. 5B, right part).

DISCUSSION

Both endogenous EGF receptors in Mv1Lu cells and transfected PDGF β-receptors in porcine aortic endothelial cells...
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were found to show decreased ligand-induced tyrosine phosphorylation in cells of dense cultures. The density-dependent difference in receptor phosphorylation was reduced by pretreatment of cells with PTP inhibitors. We therefore conclude that the difference in net tyrosine phosphorylation of PDGF-β and EGF receptors between sparse and dense cultures is caused by increased receptor-directed PTP activity in dense cultures. This was also directly demonstrated for the EGF receptor.

Earlier studies have demonstrated increased total PTP activity in lysates or membrane fractions derived from high density cultures, as well as increased levels of different transmembrane PTPs (3-8). However, no density-dependent differences in the net levels of tyrosine phosphorylation in vivo of key signaling molecules have been demonstrated. Our experiments show that one group of in vivo targets for the increased PTP activity in high cell density cultures are growth factor receptor tyrosine kinases. It is therefore possible that contact-induced growth inhibition is at least partially caused by PTP-mediated attenuation of growth factor signaling already at the level of receptor tyrosine phosphorylation. Loss of this previously unnoticed regulatory mechanism may be involved in cellular transformation.

Both EGF and PDGF β-receptors are known to be phosphorylated on a number of tyrosine residues (22, 23). Various tyrosine phosphorylated sites will activate different signaling pathways by recruiting different SH-2 domain containing proteins (reviewed in Ref. 24). Preliminary analysis of the substrate specificity of PTPs has revealed some sequence specificity. For example, when a dodecapeptide derived from the insulin receptor containing three phosphorylated tyrosine residues was used as substrate, both CD45 as well as leukocyte antigen-related phosphatase preferentially dephosphorylated the tyrosine corresponding to amino acid 1146 of the insulin receptor (25, 26). It is therefore possible that the observed density-dependent difference in receptor phosphorylation reflects specific phosphorylation of some sites, rather than complete dephosphorylation of a subset of receptors. This is a question that should be addressed in future studies.

Identification of the PTPs responsible for the increased dephosphorylation of EGF- and PDGF β-receptors in dense cultures of Mv1Lu cells and PDGF-βR-PAE cells is another important subject for future studies. When identified, they should be interesting candidates for comparative analysis regarding expression in normal cells and cancer cells. Finally, targeting of these PTPs with antisense or knock-out techniques should make it possible to formally test if those are true mediators of contact inhibition.

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Protein-tyrosine Phosphatase-mediated Decrease of Epidermal Growth Factor and Platelet-derived Growth Factor Receptor Tyrosine Phosphorylation in High Cell Density Cultures

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