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Orthovanadate Both Mimics and Antagonizes the Transforming Growth Factor β Action on Normal Rat Kidney Cells

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Normal rat kidney [NRK] cells grown in the presence of epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) have a normal phenotype and undergo density-dependent growth inhibition, whereas in the presence of multiple growth factors, density arrest is lost and the cells become phenotypically transformed. We studied the influence of the protein tyrosine phosphatase (PTPase) inhibitor sodium orthovanadate on the mitogenic stimulation of NRK cells by growth factors and on transformation-linked properties as loss of density-dependent growth inhibition and anchorage-independent growth. The fraction of cells in serum-deprived monolayer cultures that is induced to proliferate upon mitogenic stimulation by EGF or PDGF is only slightly enhanced upon addition of low concentrations (25–50 μM) of vanadate. Addition of vanadate per se induces proliferation of only a very limited amount of cells, but results in a shift of the dose-response curves for other growth factors to lower concentrations. Vanadate added in combination with EGF or PDGF is able to mimic the effect of transforming growth factor β (TGFβ) in inducing phenotypic transformation. In monolayer cultures density-dependent growth inhibition is lost and anchorage-independent proliferation is observed on dishes coated wtih poly(2-hydroxy-ethyl methacrylate) (polyHEMA). The extent of these changes is similar to that induced by TGFβ. However, the morphology of the obtained colonies in polyHEMA-coated dishes is quite different. Cells transformed by TGFβ in the presence of EGF form rather amorphous colonies, whereas in the presence of orthovanadate colonies are formed that tend to fall apart in loose cells. The effect of vanadate on cell transformation is dependent on the growth factor conditions in a bimodal way. When a suboptimal dose of growth factor(s) is used, 25 μM vanadate is very effective in preventing density-induced growth inhibition and stimulating anchorage-independent proliferation. However, the same concentration of vanadate is inhibitory when cells are maximally stimulated and antagonizes the transforming effect of TGFβ added in combination with other growth factors. It is hypothesized that vanadate acts on a set of different protein tyrosine phosphatases. Some of these are positive and others negative regulators of growth.

Normal rat kidney (NRK) cells have been widely used as a model to study the role of polypeptide growth factors in the oncogenic transformation (Van Zoelen, 1991). Grown under serum-free conditions, NRK cells show a normal phenotype in the presence of a single growth factor such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) and undergo density-dependent inhibition of growth. In the presence of multiple growth factors, however, density-dependent growth inhibition is lost. Similarly, anchorage-independent proliferation of NRK cells requires the presence of a combination of growth factors, e.g., EGF and PDGF. The response to these growth factors can be enhanced by modulating agents such as transforming growth factor-β (TGFβ) and retinoic acid. These factors, however, are not a prerequisite for phenotypic transformation including anchorage-independent growth.

Several hypotheses have been offered to explain the molecular basis of density-dependent inhibition of growth of nontransformed cells (Wieser et al., 1990; Van Zoelen, 1991). These include the autocrine production of growth inhibitory factors secreted in the medium by confluent cells; modulation of the plasma membrane potential; intercellular communication via gap junctions; specific cell-cell contacts via plasma membrane proteins also known as contactinhibins; and modulation of growth factor receptor densities and induction of protein tyrosine phosphatase (PTPase) activity. The above mechanisms are certainly not mutually

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exclusive and probably all of these processes combined will eventually lead to density-dependent growth inhibition. Recent evidence suggests that the last two mechanisms in particular are of primary importance in NRK cells (Van Zoelen, 1991).

Several classes of PTPases have been detected, both nonreceptor and receptor PTPases. The latter constitute a novel class of receptors, which in concert with the proper ligands may constitute independent regulatory pathways in the cell (Tonks and Charbonneau, 1989; Fischer et al., 1991). Structure analysis of members of this class of receptors revealed similarities with cell components known to be involved in cell-cell interactions, suggesting that they might play a role in the regulation of density-dependent inhibition of growth. In a recent study of Pallen and Tong (1991), it was shown that membrane PTPase activity of dense contact-inhibited Swiss 3T3 cells was increased eight-fold on average when compared to low-density proliferating cells. In early studies, stimulation of DNA synthesis in quiescent fibroblasts by the PTPase inhibitor orthovanadate was observed (Carpenter, 1981; Smith, 1983). Stimulation occurred in the presence of low concentrations of serum and was synergistic with the action of insulin but not with EGF (Smith, 1983). In another study (Klarlund, 1985), it was shown that NRK cells grown in the presence of normal calf serum could be transformed by vanadate as evidenced by a decreased density-dependent growth inhibition, growth in the absence of solid support and the appearance of a transformed phenotype. In later studies, however, these findings could not be confirmed (Gordon, 1991).

In this work we used a serum-free assay system as described earlier (Van Zoelen et al., 1988) to study the role of orthovanadate in the transformation of NRK cells both in the presence and in the absence of defined growth factors. We show that vanadate by itself has no growth stimulatory effects, but that in combination with either EGF or PDGF, it prevents the density-dependent growth inhibition of NRK cells. Like monolayer cultures, orthovanadate is able to induce phenotypic transformation in an anchorage-independent culture system if present in combination with EGF. In these respects, vanadate resembles TGFβ and retinoic acid.

MATERIALS AND METHODS

Cell culture

Cell culture procedures and measurement of ^{3}H-thymidine incorporation were performed as described earlier (Van Zoelen et al., 1985; 1988). Normal rat kidney cells, clone 49F, were plated at a density of 2.5×10^4/1.8 cm^2 in 24-well plates (Costar, Cambridge MA) in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Irvine, Scotland), supplemented with 10% newborn calf serum (Hyclone, Logan, UT). After incubation for 4 days, cells had reached confluency, and the medium was exchanged for 1 ml of a 1:1 bicarbonate-buffered mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Flow Laboratories), supplemented with 30 nM Na_2SeO_3 (ICN, Plainview, NY) and 10 μg/ml human transferrin (Sigma, St. Louis, MO). After 72 hr in this serum-free medium, growth factors and vanadate in the additional presence of 5 μg/ml insulin were added in 0.1 ml of binding buffer (DMEM containing 50 mM N, N-bis (2-hydroxy-ethyl)-2-amino-ethane sulphonic acid (Sigma) and 0.1% bovine serum albumin (Sigma), pH 6.8). Unless otherwise indicated, growth factors were routinely added in triplicate in the following concentrations: EGF, 5 ng/ml; PDGF type BB, 5 ng/ml, TGFβ$_1$, 2 ng/ml. ^{3}H-Thymidine (^{3}H-TdR) incorporation was measured between 4 and 17 hr and between 46 and 48 hr after mitogenic stimulation. ^{3}H-Tdr (0.5 μCi, 43 Ci/mmol, Amersham, Buckinghamshire, UK) was added in 0.1 ml Ham's F12 medium. After the indicated time intervals, cells were washed four times with ice-cold phosphate-buffered saline and incubated for 15 min with 0.5 ml methanol at room temperature. The methanol was removed and the wells were allowed to air dry. The cells were then solubilized in 1 ml 0.5 N NaOH and ^{3}TdR incorporation was measured by liquid scintillation counting. ^{3}H-TdR incorporation was measured in triplicate. Sample standard deviation was always less than 10%.

In control experiments, the amount of adherent cells was counted using the methylene blue staining procedure as described by Oliver et al. (1989).

Anchorage-independent growth

Anchorage-independent growth of NRK cells was measured on 28 cm^2 bacteriological dishes coated with poly(2-hydroxy-ethyl methacrylate) (polyHEMA, Aldrich Chemie, Steinheim, Germany) essentially as described by Folkman and Moscona (1978). Dishes were treated with 2 ml of a 6 mg/ml solution of polyHEMA in ethanol and incubated at 37°C until the solvent was fully evaporated. In order to obtain single cell suspensions from confluent NRK cultures, cells were trypsinised and replated at 50% confluency the day before the start of the experiment. After renewed trypsinisation, individual cells (5×10^5 per dish) were seeded than in 2 ml of a 1:1 bicarbonate-buffered mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Flow Laboratories), supplemented with 30 nM Na_2SeO_3 (ICN), 10 μg/ml human transferrin (Sigma), 10 μg/ml insulin, and 10% growth factor-inactivated fetal calf serum (referred to as SH-FCS). Growth factors in the same concentrations as in the monolayer culture (see above) and vanadate were added simultaneously in the same medium. Growth-factor inactivated serum was prepared by incubation of fetal calf serum (FCS, Flow Laboratories) with 100 mM dithiotreitol (Boehringer, Mannheim, Germany) followed by 5 g/lt iodoacetamide (Sigma) as described (Van Zoelen et al., 1985). After incubation for 1 week at 37°C, the cell

Abbreviations

EGF	epidermal growth factor
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
NRK	normal rat kidney
PDGF	platelet-derived growth factor
polyHEMA	poly(2-hydroxy-ethyl methacrylate)
PTPase	phosphotyrosine phosphatase
SH-FCS	FCS growth factor-inactivated by thiol treatment
TGFβ	transforming growth factor-β

suspension was collected together with two washes of the dishes in phosphate-buffered saline. The cells were collected by centrifugation and stored for 1–2 hr at –70°C. Cellular DNA content was measured according to the fluorimetric method of Labarca and Paigen (1980) using calf thymus DNA (Sigma) as a standard.

Vanadate solutions and growth factors

Stock solutions of 25 mM sodium orthovanadate (Na_3VO_4, Sigma) were either prepared fresh in distilled water without any further precautions or were adjusted to pH 10, heated, and readjusted to pH 10 as described (Gordon, 1991). The latter preparation could be stored at 4°C for several months without any loss in activity. Recombinant human PDGF type BB was a generous gift from Dr. C.-H. Heldin (Ludwig Institute, Uppsala, Sweden). Transforming growth factor-β_1 was purified from outdated human blood platelets as described (Van den Eijnden-Van Raaij et al., 1988). Receptor grade EGF was obtained from Collaborative Research (Waltham, MA), and insulin (bovine pancreas) was obtained from Sigma.

RESULTS

Influence of vanadate on the mitogenic stimulation of NRK cells with EGF or PDGF

Normal rat kidney cells grown to confluency and subsequently kept under serum-free conditions for 3 days can be induced to proliferate by the addition of EGF or PDGF (Van Zoelen et al., 1988). After one cycle of cell division, thymidine incorporation returns to control values (Fig. 1) and the cells become density inhibited. However, only a fraction of the cells (~40%; Van Zoelen et al., 1988) is stimulated under these assay conditions. This percentage can be increased by the additional presence of TGFβ or retinoic acid, which is supposed to modulate the signal of EGF positively (Van Zoelen et al., 1988). Although TGFβ and retinoic acid by themselves are not mitogenic for NRK cells, both are able to prevent the cells from becoming density-inhibited following EGF treatment.

We investigated whether the PTPase inhibitor vanadate is able to modulate the effects of EGF or PDGF. When a low concentration of vanadate is added to serum-deprived confluent NRK cells simultaneously with optimal doses of EGF or PDGF, the cumulative ^{3}H-TdR incorporation during the first day after stimulation is only slightly increased with respect to the effect of the growth factor alone (Fig. 1A). Vanadate by itself induces DNA synthesis to a very limited extent and the combined effects appear to be largely additive. However, at suboptimal doses of EGF or PDGF, the influence of vanadate on the cumulative ^{3}H-TdR incorporation can be far more than additive. The dose-response curve for the effect of the growth factor is shifted to much lower concentrations in the presence of vanadate. In the absence of vanadate, half maximal stimulation is achieved at ~0.3 ng/ml EGF, whereas vanadate lowers this concentration ~ten times (Fig. 2).

Moreover, vanadate is able to prevent density-dependent growth inhibition. After 2 days of stimulation, when DNA synthesis has stopped in the presence of either EGF or PDGF alone, ^{3}H-TdR incorporation is increased markedly in the additional presence of vanadate (Fig. 1B). This effect is more pronounced with EGF than with PDGF, probably because in contrast to EGF the availability of free PDGF decreases in time due to adsorption to proteins (E.J.J. van Zoelen, unpublished). In this respect vanadate mimics the action of TGFβ and retinoic acid (Van Zoelen et al., 1988).

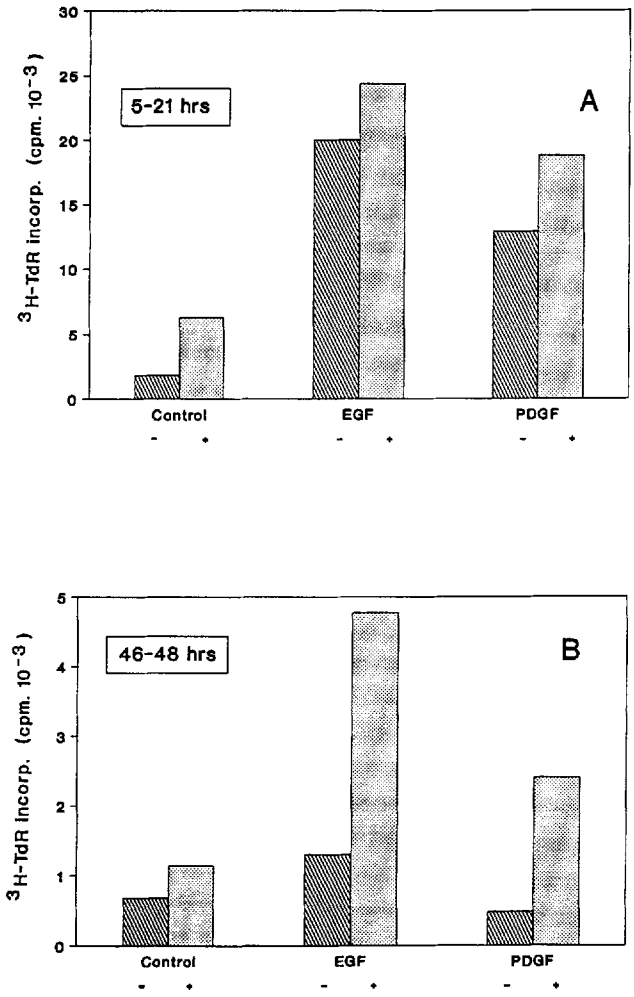

Fig. 1. Mitogenic response (**A**) and loss of density-dependent growth inhibition (**B**) of NRK cells in the presence of growth factors and vanadate. Cells are grown to confluency, incubated for 3 days in serum-free medium, and stimulated with growth factor in the absence (striped bars) and presence (stippled bars) of 25 μM vanadate. Cumulative ^{3}H-TdR incorporation is measured 15–21 hr (A) and 46–48 hr (B) after stimulation. In the control experiment, only buffer is added.

Concentration dependence of vanadate

The optimal concentration of vanadate for growth stimulation of serum-deprived NRK cells appeared to be dependent on the strength of the applied stimulus. At saturating doses of EGF or PDGF, the optimal vanadate concentration ranged between 25 and 50 μM (Fig. 3). Higher concentrations are inhibitory. As mentioned the effect of individual stimuli can be enhanced by modulating agents like TGFβ and retinoic acid or by adding multiple independently acting growth factors at the same time (Van Zoelen et al., 1988; 1991). When the

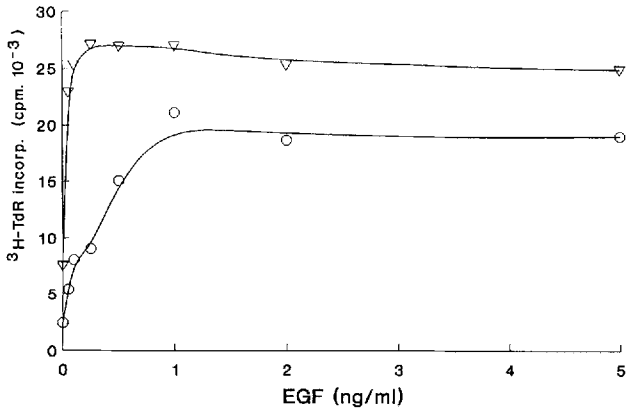

Fig. 2. Influence of vanadate on the dose response curve of EGF for the mitogenic stimulation of NRK cells. Cumulative ^{3}H-TdR incorporation is measured 5–21 hr after stimulation of NRK cells with increasing amounts of EGF in the presence (▽) or absence (○) of 50 μM vanadate.

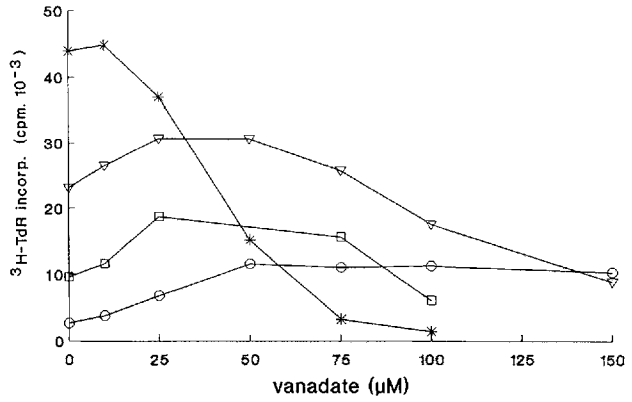

Fig. 3. Concentration dependence of the influence of vanadate on the mitogenic response of NRK to growth factors. Cells are grown to confluency, incubated for three days in serum-free medium, and stimulated with growth factors in the presence of increasing concentrations of vanadate. Cumulative ^{3}H-TdR incorporation is measured 5–21 hr after stimulation. A representative experiment out of at least ten is shown; S.D. was always <10%. (○), no growth factor added; (▽), EGF; (□), PDGF; (*), EGF + TGFβ.

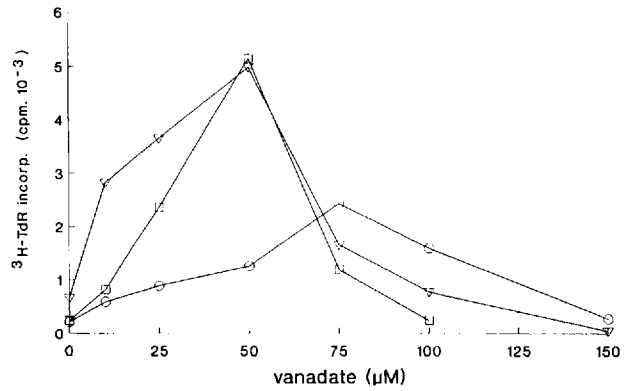

Fig. 4. Concentration dependence of the influence of vanadate on the density-dependent growth inhibition of NRK cells in the presence of EGF or PDGF. Cells are grown to confluency, incubated for 3 days in serum-free medium, and stimulated with EGF (▽) or (□) PDGF in the presence of increasing concentrations of vanadate. Cumulative ^{3}H-TdR incorporation is measured 46–48 hr after stimulation. In the control experiment (○), no growth factor is added.

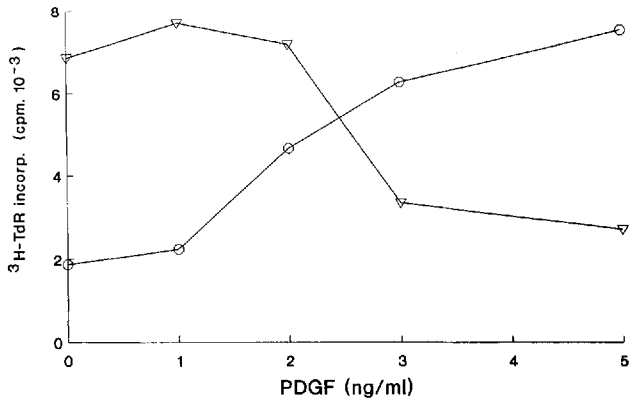

Fig. 5. Influence of vanadate on the dose-response curve of PDGF for the loss of density-dependent growth inhibition of NRK cells. Cells are grown to confluency, incubated for 3 days in serum-free medium, and stimulated with EGF (5 ng/ml) and increasing amounts of PDGF in the presence (▽) and absence (○) of 25 μM vanadate. Cumulative ^{3}H-TdR incorporation is measured 46–48 hr after stimulation.

EGF signal is amplified by TGFβ, vanadate—even at very low concentrations—is a strong inhibitor of cell proliferation. However, in the absence of added growth factors, no inhibition at all is observed up to 150 μM (Fig. 3).

Similar dual effects of vanadate dependent on the extent and the nature of the applied stimulus are observed with respect to the prevention of density-dependent growth inhibition as illustrated in Figures 4 and 5. A bell-shape curve is obtained when the concentration of vanadate is varied with maximal stimulatory activities at ~50 μM in the presence of either EGF or PDGF, and at 75 μM in the absence of these growth factors (Fig. 4). In the experiment shown in Figure 5, NRK cells are stimulated with a optimal dose of EGF in the additional presence of increasing amounts of PDGF. The ^{3}H-TdR incorporation is measured 46–48 hr after mitogenic stimulation, which is well after completion of the first round of cell division. In the absence of PDGF, cells have stopped dividing, whereas PDGF induces a proliferative activity dependent on its concentration. At low concentrations of PDGF proliferation is stimulated by the presence of 25 μM vanadate, whereas the same concentration is inhibitory at higher concentrations of PDGF.

It might be argued that the inhibitory effects of vanadate represent a general cytotoxic mechanism of action, which is independent from the presence of specific growth factors, but may relate to other known cellular effects of vanadate (Gordon, 1991). Toxic effects result in rounding up of cells and a subsequent cell loss during the washing procedure. Although it cannot be excluded that this accounts at least partly for the effects of vanadate in the higher concentration range, the data shown

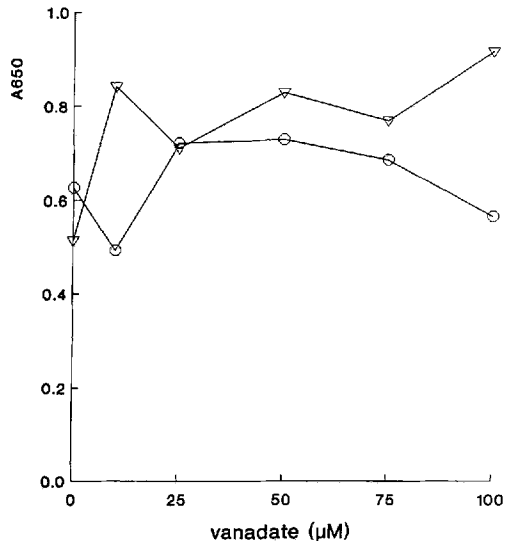

Fig. 6. Influence of vanadate on cell numbers. Cells are grown to confluency, incubated for 3 days in serum-free medium, and stimulated with EGF (○) or EGF/TGFβ (▽) in the presence of increasing concentrations of vanadate. After 21 hr incubation, the medium is removed and the wells are washed four times with phosphate-buffered saline. The relative number of adherent cells (A_{650}) is measured with the methylene blue staining method.

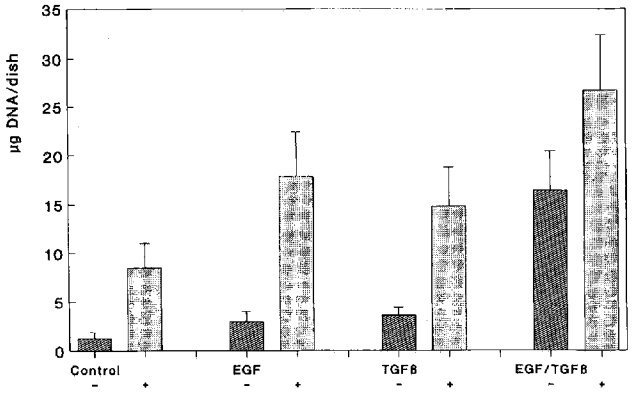

Fig. 7. Stimulation of anchorage-independent growth of NRK cells by vanadate. Cells were seeded in bacterial dishes coated with poly-HEMA in the presence of growth factors with (stippled bars) and without (striped bars) 25 μM vanadate. After 1 week of culture, cells were collected and total cellular DNA was measured. In the control experiment, no growth factors were added. Bars represent the mean ± S.D. for three separate experiments.

in Figures 3–5 argue for a regulatory mechanism of vanadate at relatively low concentrations on the signal transduction pathway, which is triggered by the particular growth factor. The same concentration of vanadate is inhibitory in some conditions and stimulatory in others. To directly exclude the loss of cells, the amount of adherent cells is counted after the washing procedure with the methylene blue staining method of Oliver et al. (1989) (Fig. 6). Neither in the presence of EGF nor EGF/TGFβ significant cell loss is found up to 100 μM vanadate.

Anchorage-independent growth

Anchorage-independent growth of NRK cells can be induced by adding multiple polypeptide growth factors (Van Zoelen et al., 1986, 1989, 1988; Van Zoelen, 1991). Whereas EGF and TGFβ per se are unable to induce this aspect of phenotypic transformation, proliferation is provoked in the presence of both growth factors. Cell growth in the absence of solid support is usually examined by the ability to form colonies is soft agar, but can also be measured in suspension on dishes coated with polyHEMA, which prevents cells from adherence (Folkman and Moscona, 1978). In the latter method the extent of total cell proliferation is measured, rather than the proportion of colony formation or the extent of proliferation per colony as in soft agar culture. We have chosen polyHEMA-coated dishes to examine the ability of vanadate to transform NRK cells both in the presence and the absence of growth factors. Therefore, growth factor-inactivated serum was used in the medium in order to exclude the interference of serum-derived growth factors.

In accordance with studies in soft agar (Van Zoelen et al., 1986, 1988, 1989, 1991) EGF and TGFβ by itself are unable to induce significant cell proliferation, but produce a significant response when added together (Fig. 7). In contrast vanadate per se provokes a significant anchorage-independent growth, whereas in combination with either EGF or TGFβ an even higher response is observed. In combination with EGF the potencies of vanadate and TGFβ to induce cell growth are comparable. Cell growth in the presence of EGF and TGFβ together can even be increased further by the additional presence of vanadate. It should be noted, however, that omission of insulin from the culture medium abrogates all effects of vanadate. No transformation whatsoever can be observed in the absence of insulin (results not shown). The concentration dependence of the effect of vanadate is similar in anchorage-independent growth and in the prevention of density-dependent growth arrest. Optimal concentrations for growth in polyHEMA cultures also range between 25 and 50 μM, at higher concentrations inhibitory effects are observed (results not shown).

Although the influence of EGF/vanadate and EGF/TGFβ on cell growth, determined by measuring total cellular DNA, is comparable, there is a remarkable difference in the morphology of the colonies (Fig. 8). Colonies formed in the presence of EGF/TGFβ are rather dense and amorphous, whereas in the presence of vanadate cells are rounded and colonies tend to fall apart in single cells. This phenomenon is seen in all cultures in which vanadate is present, both in the presence and in the absence of additional growth factors (except insulin).

DISCUSSION

Normal rat kidney cells are basically nontumorigenic cells, which are unable to grow in the absence of externally added growth factors, undergo density-dependent growth inhibition, and do not proliferate in the absence of solid support. However, the density-dependent growth inhibition can be prevented or overcome by the addition of multiple growth factors (Van Zoelen et

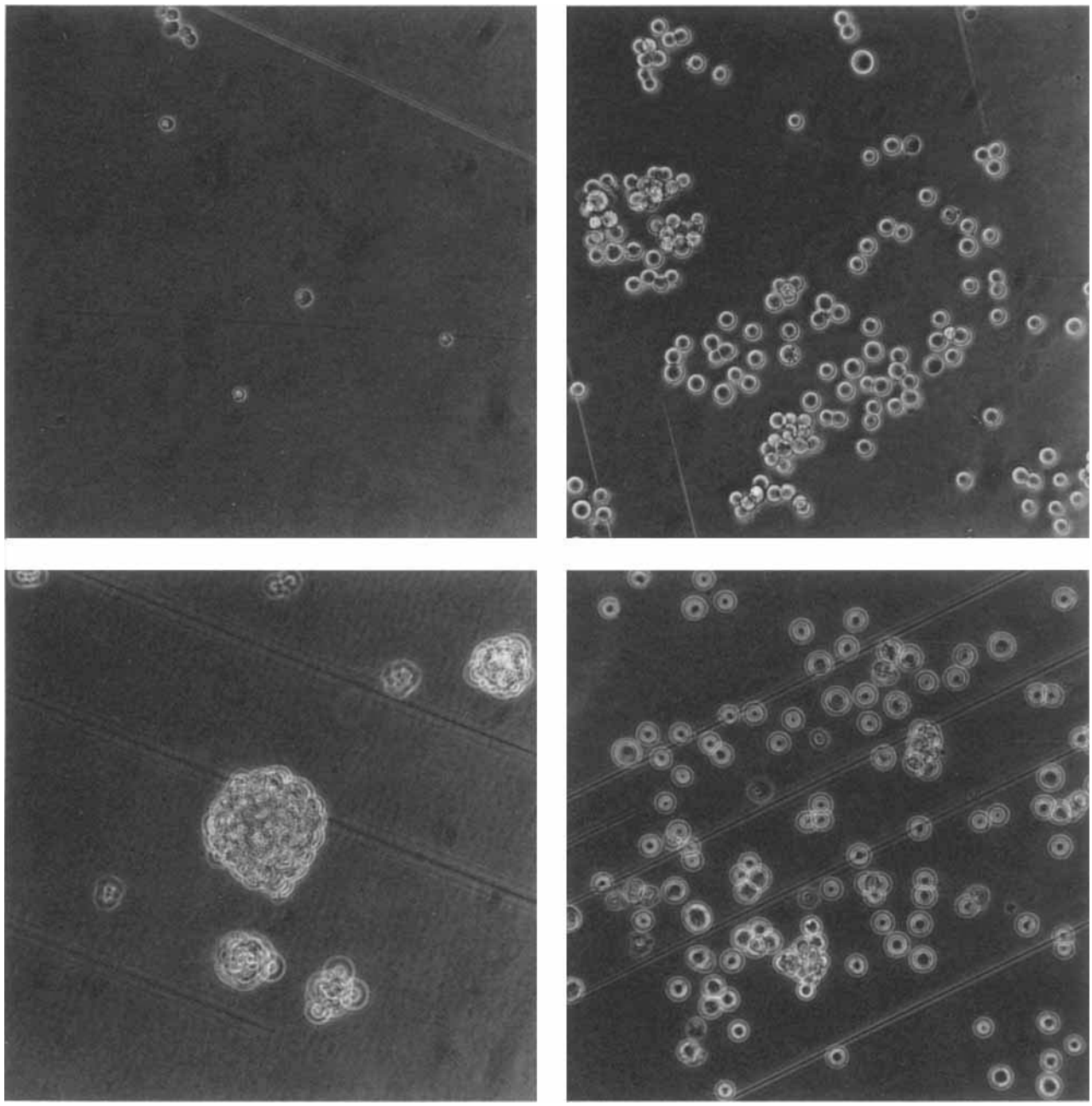

Fig. 8. Influence of vanadate on the morphology of colonies of NRK cells in polyHEMA cultures. Cells were cultured in bacterial dishes coated with polyHEMA in the presence of EGF **(upper left)**, EGF and 25 μM vanadate **(upper right)**, EGF and TGFβ **(lower left)**, and EGF, TGFβ, and 25 μM vanadate **(lower right)**. Cultures were photographed 1 week after inoculation.

al., 1988, 1991). Similarly, anchorage-dependent growth can be induced in growth factor defined conditions. As loss of density-dependent growth inhibition and anchorage-independent growth are fundamental characteristics of transformed cells, NRK cells offer a good model to study the growth factor requirements of phenotypic transformation and the molecular mechanisms of growth factor action. It is clear now that the phosphorylation of tyrosyl residues plays a key role in these mechanisms. Since a regulatory role for PTPases have been suggested in counteracting the effects of tyrosine kinases (Tonks and Charbonneau, 1989; Fischer et al., 1991; Pallen and Tong, 1991), we decided to study the role of PTPase activities in the transformation of NRK cells by using the inhibitor orthovanadate.

Stimulation of confluent NRK cells, which are made quiescent by serum-deprivation, by EGF induces cells to undergo one round of cell division. However, only

~40% of cells are sensitive to the action of EGF under these conditions (Van Zoelen et al., 1988, 1991). It has been hypothesized that the level of EGF receptors in this particular cell type is critical as it determines the change of individual cells to start proliferation upon stimulation with EGF (E.J.J. van Zoelen, unpublished). The fraction of cells that enter the cell cycle can be increased by adding modulating agents like TGFβ or retinoic acid, which among other effects may act by increasing the level of EGF receptors (Assoian et al., 1984; Van Zoelen et al., 1986; Thompson et al., 1988). In several cell lines, receptor levels have been shown to decrease with increasing cell densities and a causal relationship with density-dependent growth inhibition has been suggested (Rizzino et al., 1988; Lichtner and Schirrmacher, 1990; Rizzino et al., 1990).

In the present study we show that in contrast to TGFβ, vanadate hardly increases the proportion of serum-deprived quiescent cells, which enter mitosis after stimulation with an optimal dosis of EGF. However, the effect of low doses of the growth factor is strongly potentiated by vanadate, indicating that it optimizes the growth factor signal in those cells that are capable of generating a signal sufficient to induce proliferation. Moreover, in these cells the density-dependent inhibition is prevented by the addition of vanadate. In these cells vanadate may act by inhibiting a PTPase that is responsible for the dephosphorylation of autophosphorylated EGF receptors or tyrosyl-phosphorylated substrates downstream in the signal transduction pathway. As a consequence the feedback regulation of the signal is impaired, resulting in a prolonged stimulatory effect of EGF in sensitive cells. It is conceivable that in particular long-term mitogenic effects of EGF can be sustained by inhibition of the dephosphorylation of the receptor or its substrates, resulting in prevention of the density-dependent growth inhibition. The presence of a vanadate-sensitive PTPase responsible for the rapid dephosphorylation of autophosphorylated EGF receptor has been suggested before (Rotin et al., 1992), and recently a soluble PTPase has been cloned forming high-affinity complexes with the activated EGF receptor (Shen et al., 1991), also giving support to the notion that tyrosine kinase associated signals might be down regulated via PTPases. Inversely, feedback regulation may be bilateral as tyrosyl phosphorylation of both receptor (Stover et al., 1991) and nonreceptor (Shen et al., 1991) PTPases have been reported. In Swiss 3T3 cells, membrane PTPase activity is upregulated with increasing cell density, suggesting that it plays a role in the growth arrest of dense cultures (Pallen and Tong, 1991).

In addition to positive regulatory influences of vanadate on mitogenic stimulation of serum-deprived cells and on the prevention of density-dependent growth inhibition, we also found growth inhibitory effects dependent on its concentration and the growth factor conditions. These negative effects cannot be easily explained by general toxic effects via mechanisms independent of PTPase inhibition. In vivo effects of vanadate on several cellular processes, including increase in intracellular pH and Ca^{2+} and action as an intracellular redox system, have been reported (Gordon, 1991) as well as general toxicity resulting in cell death (Klarlund, 1985). However, the present results argue against an effect of vanadate independent of the activation of tyrosine-associated growth factor receptors, as the same concentration of vanadate is stimulatory in some conditions and inhibitory in others. Furthermore, only at concentrations of vanadate above 100 μM, we found a significant loss of cells due to cell death. Therefore, we suggest that alternatively the bimodal effect of vanadate may be explained by the inhibition of multiple PTPases with different affinites for vanadate.

In contrast to the down-regulation of signals associated with increased tyrosine kinase activity, PTPases may at the same time activate cellular tyrosine kinases. A family of nonreceptor tyrosine kinases consisting of at least seven members related to the cellular src protein are supposed to play important roles in the transduction of proliferative signals both in transformed cells and in normal fibroblasts (Brickell, 1991). All members of the src-family are regulated by phosphorylation of a common carboxy-terminal tyrosine residue, e.g., Tyr-527 in c-src. Phosphorylation of this residue results in inactivation of the kinase activity and thus offers a potential activating mechanism by a PTPase. Indeed, the involvement of a regulatory PTPase in the activation of src-related kinases has been well documented. In lymphocytes an integral membrane receptor with associated tyrosine phosphatase activity (CD-45) positively regulates tyrosine kinase-associated signals operating through src-like proteins (Srivastava et al., 1991; Trowbridge, 1991). Inhibition of these activities with vanadate would block the signal.

As mentioned, the inhibitory effect of vanadate appeared to be dependent on the growth factor conditions. At suboptimal doses of one particular growth factor, low concentrations of vanadate are stimulatory, whereas the same concentrations are inhibitory at saturating doses of growth factor. Furthermore, when a signal transduction pathway is optimally triggered, e.g., when the EGF signal is enhanced by adding TGFβ, vanadate is inhibitory even at low concentrations. These differences in dose dependency of vanadate make sense if receptor tyrosine kinases each induce the activity of a specific PTPase involved in the transduction of their signal. Indeed, tyrosine phosphorylation of the receptor PTPase CD45 has been observed in response to activation of Jurkat T cells (Stover et al., 1991).

Proliferation in the absence of solid support is a major characteristic of transformed tumorigenic cells. In the present study we show that the addition of vanadate induces the anchorage-independent proliferation of NRK cells. Furthermore, whereas EGF and TGFβ on their own are unable to support growth, both factors work synergistically with vanadate. The proliferative response to the combination EGF/TGFβ can be enhanced ever further by the additional presence of vanadate. In all cases the presence of insulin is obligatory, suggesting that the signal transduction pathways triggered by insulin and EGF act cooperatively and that vanadate is able to enhance both signals. The same growth factors were required to prevent density-dependent growth inhibition in monolayer culture and to induce anchorage-independent growth. In addition, the dose-dependent effects of vanadate are similar. This

suggests that the same molecular mechanisms might be involved in its bimodal modulation of the respective signal transduction pathways.

There is some evidence that cells with a transformed phenotype can grow in the absence of solid support because of the production of extracellular matrix proteins like fibronectin (Ignotz and Massagué, 1986; Allen-Hoffmann et al., 1988). Indeed, both fibroblasts transformed by overexpression of the proto-oncogene c-sis (Allen-Hoffmann et al., 1990) and NRK cells transformed by the addition of TGFβ (Rizzino, 1988) produce increased amounts of extracellular matrix proteins. In both cases the anchorage-independent growth is blocked by the addition of RGD peptides. However, we now show that NRK cells transformed in the presence of vanadate show a quite different morphology from that obtained in the presence of EGF/TGFβ. Cells are rounded up and colonies tend to fall apart into single cell suspensions. This finding strongly argues against the formation of extracellular matrix proteins and consequent cell-cell interactions via adhesion receptors as a prerequisite for anchorage-independent growth. Thus it can be concluded that the production of extracellular matrix proteins is not obligatory for anchorage-independent growth.

The results of the present study show that in the appropriate conditions the PTPase inhibitor vanadate is able to induce all the characteristics of transformed cells, including the proliferation in absence of serum-derived growth factors, the loss of density-dependent growth inhibition, and growth in the absence of solid support. Obviously NRK cells possess PTPase activity that can regulate signals transmitted by tyrosine kinase associated receptors and thus potentially plays a role in the process of phenotypic transformation.

Vanadate mimics TGFβ for the greater part in the induction of phenotypic transformation. However, whereas TGFβ is supposed to upregulate receptor densities and thus to enhance incoming signals, vanadate in contrast does not amplify the initial signal but prevents its down-regulation. This model predicts that after down-regulation of EGF receptor densities, i.e., in density-inhibited cells, vanadate is unable to restimulate proliferation in contrast to TGFβ. Indeed, vanadate appeared to be ineffective in overcoming density-dependent growth arrest (results not shown), whereas TGFβ restimulates cell growth to a large extent (Van Zoelen, 1991).

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