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Stimulation of glycolysis and amino acid uptake in NRK-49F cells by transforming growth factor β and epidermal growth factor

(tumor glycolysis/methionine/methylaminoisobutyrate/system A)

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ABSTRACT **Glycolysis in normal resting rat kidney cells (NRK-49F) was stimulated by a 2-hr exposure to transforming growth factors prior to assay. Transforming growth factor β (TGF-β) was effective when added alone, and further addition of epidermal growth factor (EGF) had little effect. The stimulation by TGF-β was abolished when cycloheximide was present during the incubation, suggesting that protein synthesis is required for the effect. Incubation of the cells with 25 mM methionine abolished the stimulation of glycolysis by TGF-β. The uptake of methylaminoisobutyrate via system A was stimulated by either TGF-β or EGF. The >3-fold stimulation of uptake by 1 ng of pure TGF-β per ml was usually somewhat enhanced on addition of 0.5 ng of EGF per ml. Moreover, an antiserum against EGF receptor partially depressed the response to TGF-β, suggesting some overlapping interactions of EGF and TGF-β.**

The discovery of multiple oncogenes capable of malignant transformation of cells grown in tissue culture (1) represents an important advance in the genetic analysis of tumorigenesis. The discovery of multiple transforming growth factors, TGF-α and TGF-β, which together induce in normal cells phenotypical changes that usually characterize tumor cells, has opened up new avenues of approach to the biology and biochemistry of malignant transformation (2). While genetic analyses point the way to gene products and their functional expressions, the study of TGFs allows us to search more specifically for the biochemical changes induced by the individual factors and to determine the relationship of these changes to biological manifestations.

Among the biochemical features that are frequently found in tumors is an enhanced rate of aerobic glycolysis (3) and increased uptake of amino acids (4), often via system A (5–7). Analyses of the causes of the increased glycolysis of tumor cells point to an enhanced availability of P_i and ADP (8). Accelerated hydrolyses of ATP by an inefficient Na^+,-K^+-pump, by mitochondrial ATPase, or by an unknown, quercetin-sensitive ATPase have been proposed to take place in different tumor cell lines. The enhanced Na^+-dependent uptake of methylaminoisobutyrate (MeAIB) by system A in a transformed Madin–Darby canine kidney (MDCK) cell line was shown not to be a consequence of an increased growth rate and was not affected by amino acid starvation (6). The increased rate of MeAIB uptake seen in normal MDCK cells on removal of prostaglandin E_1 and the inhibition of MeAIB uptake in plasma membrane vesicles from 3T3 cells by cAMP-dependent protein kinase (9) suggest a regulation by a phosphorylation–dephosphorylation mechanism.

We have observed recently that glycolysis in a variety of normal cell lines was markedly stimulated in the presence of a crude preparation of TGF-β from human placenta during a 60-min assay (10). In this paper we report that both partly purified and pure preparations of TGF-β from human platelets stimulate glycolysis as well as the uptake of MeAIB into normal rat kidney cells (NRK-49F) after an incubation period of 2–4 hr. In an accompanying paper, an independent study is reported on the stimulation of glucose uptake into 3T3 cells by epidermal growth factor (EGF) and TGF-β (11).

MATERIALS AND METHODS

NRK-49F cells were obtained, grown in 35-mm Falcon plates, and analyzed for lactate production after starvation for 16 hr in 0.5% calf serum as described (10). Exposure to growth factors and methionine was as described in the figure legends. EGF was obtained from Collaborative Research (Waltham, MA). Crude TGF-β was extracted from outdated human platelets (12). Samples of pure TGF-β were generous gifts from A. Roberts and J. Massagué. Other growth factors and chemical reagents were purchased from Sigma. [^{14}C]MeAIB was purchased from New England Nuclear, and [^{3}H]methionine and [^{3}H]alanine were from Amersham. Transport assays were performed basically as described (6). [^{3}H]Alanine uptake was measured after 2 min at pH 6.0 in the presence of 1 mM MeAIB to ensure that alanine uptake occurred via system ASC and not via system A. [^{3}H]Methionine uptake was measured after 1 min with *N*-methyl-D-glucosamine substituted for NaCl to ensure uptake via system L (13). After washing, the cells were dissolved in 2 ml of 0.2 M NaOH/1% sodium dodecyl sulfate and assayed for radioactivity in 10 ml of Liquiscint (National Diagnostics, Somerville, NJ).

RESULTS

We have reported previously (10) that crude preparations of TGF-β from human placenta stimulate glycolysis when added to a variety of normal cells grown in tissue culture after "starvation" for 16 hr in the presence of 0.77% fetal calf serum. We now observe with preparations of TGF-β from human platelets that stimulation of glycolysis in NRK-49F cells starved for 16 hr in 0.5% calf serum required incubation with growth factors prior to the initiation of the assay. It can be seen from Fig. 1 that a significant stimulation of glycolysis occurred following an incubation with TGF-β and EGF, which peaked at ≈2 hr. After reaching the maximal level, the rate of lactate formation declined slowly in both the presence and absence of growth factors. In these experiments both TGF-β and EGF were used, but, as will be shown below, pure TGF-β stimulated glycolysis in the absence of EGF. It can also be seen from Fig. 1 that 25 mM methionine markedly inhibited glycolysis in the presence of growth factors but not in their absence. An inhibition of

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Abbreviations: TGF-α and TGF-β, transforming growth factors α and β; EGF, epidermal growth factor; MeAIB, methylaminoisobutyrate; MDCK, Madin–Darby canine kidney.

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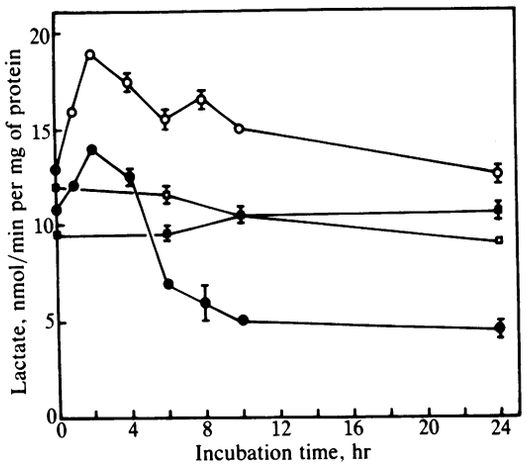

FIG. 1. Time course of TGF-β-stimulated and methionine-sensitive glycolysis. NRK-49F cells were plated at a density of 1–2 × 10^5 per 35-mm dish in 2 ml of Dulbecco's modified Eagle's medium (DME medium) and 10% calf serum. Confluent cells were washed with DME medium and incubated at 37°C with 2 ml of DME medium and 0.5% calf serum. After starvation for 18 hr, 25 mM methionine was added to the appropriate samples. Two hours after addition of methionine, partially purified platelet TGF-β (1 μg/ml) and EGF (10 ng/ml) were added and lactate production was assayed as described (10) at the indicated times. Growth factors and methionine were present during the assay in the appropriate samples at the same concentrations as during the preincubation. ○, TGF-β and EGF; ●, TGF-β, EGF, and methionine; □, control; ■, control and methionine.

glycolysis of tumor cells by methionine will be described elsewhere (unpublished data). It is noteworthy that the rate of glycolysis in the presence of growth factors and methionine was consistently lower than in the control with methionine alone, indicating that the growth factors have induced a shift in the contribution to the ADP and P_i pool toward a methionine-sensitive mechanism.

Some variability was observed with respect to the time point of maximal glycolysis. When maximal stimulation of glycolysis by the growth factors was observed within 2 hr, a mild stimulation was noted even in the presence of methionine (Fig. 1). However, no stimulation was observed in the presence of methionine by TGF-β when the peak of glycolysis was reached only after 4–6 hr. This is consistent with the observation that the effect of methionine usually requires longer periods of incubation than the effect of growth factors. The effect of EGF alone was more variable than that of TGF-β. Sometimes EGF alone stimulated glycolysis, but, as a rule, the effect of TGF-β was more pronounced than that of EGF, and the addition of EGF resulted in only little further stimulation. None of several other growth factors tested, including insulin, nerve growth factor, prostaglandin E_1, triiodothyronine, and hydrocortisone, gave rise to methionine-sensitive glycolysis.

It can be seen from Fig. 2 that the effect of the growth factors was completely abolished in the presence of cycloheximide. Actinomycin D (not shown) also prevented the stimulation. These findings suggest that the stimulation of glycolysis by TGF-β requires protein synthesis, but it remains to be established whether or not a specific protein is involved either directly or indirectly. Methionine inhibited glycolysis only in the absence of cycloheximide. Other amino acids that have a high affinity for system A of amino acid transport also inhibited glycolysis, as will be documented elsewhere. These observations suggest that a specific effect of TGF-β could somehow involve system A for amino acid transport.

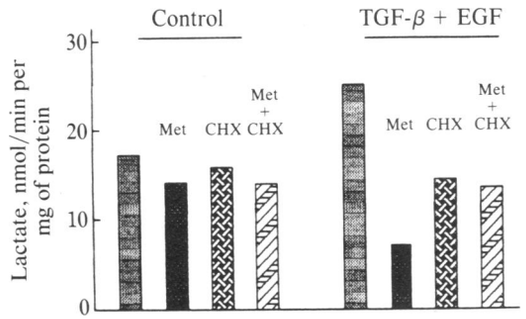

FIG. 2. Cycloheximide sensitivity of growth factor-stimulated, methionine-sensitive glycolysis. NRK-49F cells were plated at a density of 1–2 × 10^5 cells per 35-mm dish in DME medium and 10% calf serum. Confluent cells were washed with DME medium and incubated at 37°C with 2 ml of DME medium and 0.5% calf serum. After starvation for 16 hr, L-methionine (Met) (25 mM), partially purified platelet TGF-β (200 ng/ml), EGF (10 ng/ml), and cycloheximide (CHX) (25 μg/ml) were added to the appropriate plates and incubated for 6 hr, and lactate production was assayed. Growth factors, L-methionine, and cycloheximide concentrations were maintained in the appropriate samples as indicated above during the assay for lactate production.

In view of these considerations it was of interest to examine the effect of the growth factors on the uptake of amino acids via system A. As can be seen from Fig. 3, both EGF and TGF-β stimulated the uptake of MeAIB, with TGF-β showing a more pronounced effect. EGF and TGF-β together stimulated more than either alone after incubation for 24 hr (see below). As in the case of glycolysis, stimulation was observed only after some hours of incubation with TGF-β prior to the measurement of transport. In this case also, cycloheximide or actinomycin D abolished the stimulation. A titration of the concentration of TGF-β on glycolysis and MeAIB uptake is shown in Fig. 4. In this case a homogeneous preparation of TGF-β from human platelets was used. It can be seen that glycolysis was maximally stimulated at 500 pg/ml, whereas at 1000 pg/ml the stimulation was less than maximal. In another experiment, 200 pg/ml was optimal and 500 pg/ml was suboptimal. There was only little further enhancement when EGF was added together with TGF-β (Fig. 4 *Upper*). Pure TGF-β stimulated

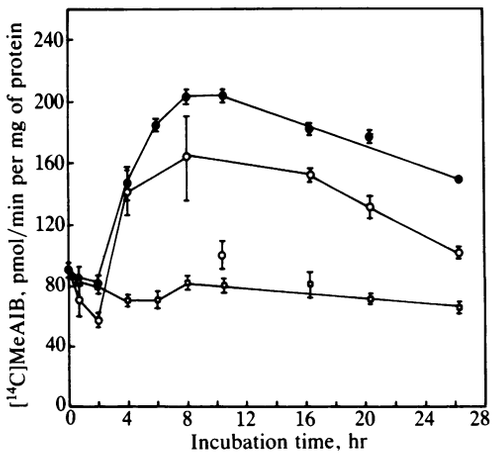

FIG. 3. Time course of the stimulation of MeAIB uptake by TGF-β (●) and EGF (○). NRK-49F cells were plated at a density of 1 × 10^5 per 35-mm dish in DME medium and 10% calf serum and grown to confluence. Partially purified TGF-β (0.5 μg/ml) or EGF (1 ng/ml) was added at zero time and [^{14}C]MeAIB uptake was measured at the indicated times. □, Control.

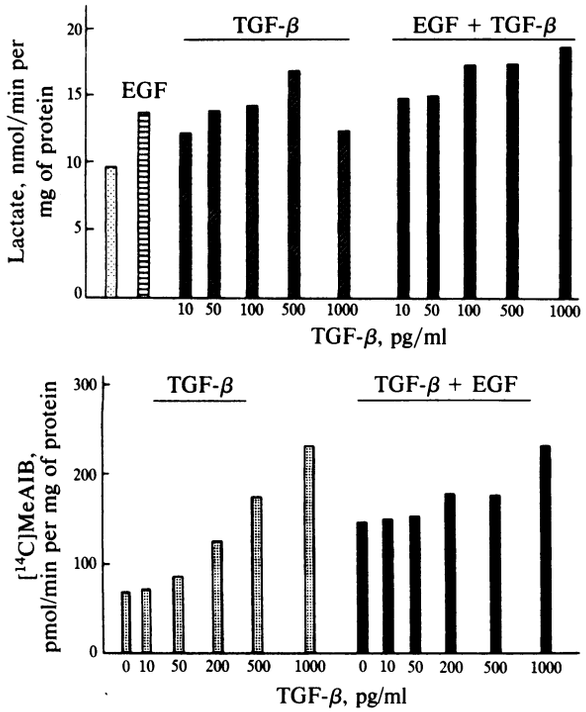

FIG. 4. Concentration dependence of the stimulation of glycolysis and MeAIB uptake by pure TGF-β. (*Upper*) NRK-49F cells were plated at a density of 2 × 10^5 per 35-mm dish in DME medium and 10% calf serum. Cells were washed with DME medium and incubated at 37°C with 2 ml of DME medium and 0.5% calf serum. After starvation for 16 hr, pure platelet TGF-β was added at the concentrations indicated, in the presence and absence of EGF (1 ng/ml), incubated for 6 hr, and assayed for lactate production. Growth factors were maintained at the above concentrations during the assay for lactate production. ▨, Control. (*Lower*) NRK-49F cells were plated at a density of 2 × 10^5 per 35-mm dish in DME medium and 10% calf serum and grown to confluence. Pure TGF-β was added to the growth medium at the indicated concentrations, in the presence or absence of EGF (0.5 ng/ml). After 15 hr, [^{14}C]MeAIB uptake was measured.

MeAIB uptake severalfold (Fig. 4 *Lower*). In this particular experiment no enhancement was observed when EGF was added as well.

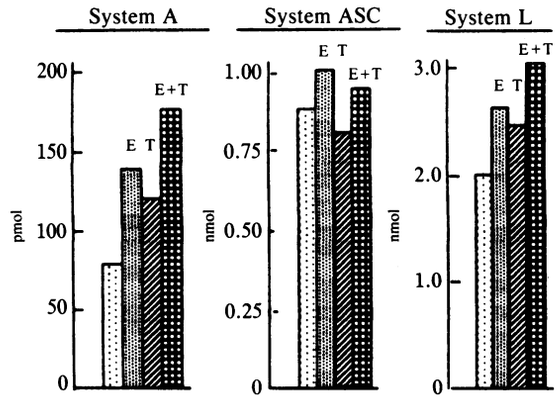

FIG. 5. Comparison of the effects of purified TGF-β and EGF on amino acid transport by systems A, ASC, and L. NRK-49F cells were plated at a density of 2 × 10^5 per 35-mm dish in DME medium and 10% calf serum and grown to confluence. TGF-β (T) (0.2 ng/ml) or EGF (E) (0.5 ng/ml) was added to the growth medium and uptake of amino acids was measured after 15 hr as described under *Materials and Methods*. Activity is reported in terms of pmol or nmol per min per mg of protein. ▨, Control.

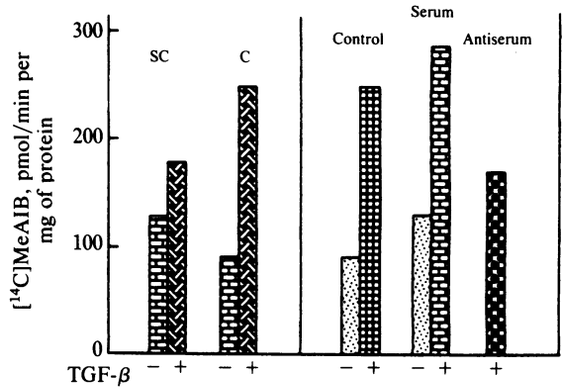

FIG. 6. (*Left*) Comparison of the stimulation of MeAIB uptake by TGF-β in subconfluent (SC) and confluent (C) cells. NRK-49F cells were plated at a density of 1–3 × 10^5 per 35-mm dish in DME medium and 10% calf serum and grown either to a subconfluent density or to confluence, as indicated. Partially purified TGF-β was added at 0.2 μg/ml and [^{14}C]MeAIB uptake was assayed after 10 hr. (*Right*) Effect of anti-EGF receptor antiserum on MeAIB uptake in the presence of TGF-β. NRK-49F cells were plated at a density of 3 × 10^5 per 35-mm dish in DME medium and 10% calf serum. Anti-EGF receptor antiserum 451 (14) was added at a 1:100 dilution. After incubation for 1½ hr, partially purified TGF-β was added at 0.2 μg/ml. [^{14}C]MeAIB uptake was assayed after 10 hr.

In the experiment shown in Fig. 5 (system A) the uptake of MeAIB was most rapid when both EGF and TGF-β were added. System ASC ([^{3}H]alanine uptake at pH 6 in the presence of MeAIB) was only marginally stimulated by EGF. Both EGF and TGF-β stimulated system L to a small extent, but the highest transport activity was observed when both growth factors were present. This stimulation that occurs in the absence of Na^+ was unexpected since in different transformed cells only Na^+-dependent transport either via system A (6) or systems A and ASC (7) has been reported to be elevated in comparison to the nontransformed cells.

Maximal stimulation of MeAIB uptake by TGF-β was seen in cells that were allowed to reach confluency (Fig. 6 *Left*). EGF alone also stimulated the uptake of MeAIB but not as markedly as TGF-β. Moreover, an antibody against EGF receptor (no. 451) that interferes with the binding of EGF partially inhibited the stimulation by TGF-β, suggesting that some EGF was available even when none was added or that a conformational change in the EGF receptor modulates the effect of TGF-β.

DISCUSSION

In this paper we have recorded observations on the stimulation by TGF-β of glycolysis and of MeAIB uptake in normal rat kidney cells. EGF had similar but less pronounced effects. The stimulation of glycolysis was most marked after incubating serum-starved cells with the growth factors for 1 to several hours prior to the addition of glucose. This is in contrast to the stimulation of glycolysis observed previously with crude preparations of placental TGF-β that did not require incubation prior to addition of glucose. It will be of interest to explore the possibility that the crude placental preparations contain additional factors that either greatly accelerate the effect of TGF-β or independently stimulate glycolysis. The observation that inhibition of protein synthesis by cycloheximide eliminated the stimulation suggests that protein synthesis is involved directly or indirectly in the phenomenon of accelerated glycolysis and MeAIB uptake.

It was emphasized previously (8) that for each lactate formed from glucose, one ATP is generated and must be

cleaved to ADP and P_i to maintain steady-state glycolysis. Since on addition of TGF-β there is an increase in lactate production, there must be a parallel increase in ATP hydrolysis. A less likely possibility is that the supply of P_i comes either from increased entry of P_i or increased hydrolysis of internal precursors—e.g., phosphoproteins. In that case, ADP must arise from ATP transfer reactions catalyzed by, for example, hexokinase or phosphofructokinase. Such a mechanism could be operative for short time periods until the accumulation of hexose phosphates limits further glucose utilization.

The question of which ATP hydrolyzing enzymes contribute significantly to glycolysis has remained unanswered for many tumor cells. In Ehrlich ascites tumor cells the Na^+,K^+-ATPase contributes about half of the P_i/ADP pool (15). In a few cell lines rutamycin inhibits glycolysis, implicating mitochondrial ATPase. In many cell lines the contributors to the P_i/ADP pool are unknown since glycolysis is resistant to both ouabain and rutamycin (8). In some, including NRK-49F cells, rutamycin actually stimulates glycolysis (by eliminating the Pasteur effect), showing that the mitochondria contribute very significantly to the generation of ATP from ADP and P_i. In any case, the lack of inhibition by rutamycin and by ouabain eliminates both mitochondrial and Na^+,K^+-ATPase activities as significant contributors to the P_i/ADP pool in these cells.

The stimulation of amino acid uptake by TGF-β is in line with previous observations mentioned earlier that transformed cells have an enhanced uptake of AIB compared to nontransformed cells. The curious findings that substrates, especially methionine, of the Na^+-dependent system A of amino acid transport strongly counteracted the enhanced glycolysis induced by TGF-β and inhibited glycolysis of many transformed cell lines may be interesting clues pointing to some common denominator. We consider, among the possibilities, a system A-associated ATPase activity, which may so far have escaped detection.

In the case of glycolysis we have observed only little or no stimulation by EGF when an excess of TGF-β was added. This is in contrast to the stimulation of glucose uptake (11). These results could imply that the stimulation of lactate production is a simpler process, whereas growth in soft agar and stimulation of glucose uptake require a more complex interaction between the two growth factors. Although stimulation of glycolysis was best seen in serum-starved cells, the stimulation of amino acid uptake by the growth factors was optimal in cells grown in medium containing high serum levels. The differences may be quantitative and blockage of the EGF receptor by an antibody that interferes with EGF binding (11) may reveal a role of EGF receptor that could not be observed because residual EGF or TGF-α in the medium may have provided sufficient growth factor to stimulate glycolysis (see Fig. 6).

Of particular interest are observations that will be documented elsewhere that several transformed cell lines exhibit methionine-sensitive glycolysis similar to that observed in normal cells exposed to TGF-β. The finding that glycolysis in TGF-β-treated cells in the presence of methionine is even lower than in control cells suggests that TGF-β induces a shift in the contribution to the P_i/ADP pool from a methionine-insensitive to a methionine-sensitive mechanism.

Since the effect of methionine is readily reversible and since MeAIB, a substrate of the system A that is not metabolized, is also effective as an inhibitor of glycolysis, it seems likely that these compounds act directly rather than via a metabolite that interacts covalently. It also opens the possibility that an analog that can bind covalently may be more effective. Alanylcholoromethyl ketone (donated by C. Kettner) inhibited system A transport only little, whereas, among several methionine analogs tested, cycloleucine and methyl- or ethylcysteine showed promise as effective inhibitors of both MeAIB uptake and glycolysis.

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