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Glycolysis and methylaminoisobutyrate uptake in rat-1 cells transfected with *ras* or *myc* oncogenes

(transforming growth factors/methionine/system A/ATPase/amino acid transport)

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ABSTRACT **A high rate of aerobic glycolysis was catalyzed by rat-1 cells transfected with a *ras* oncogene (*ras* cells); rat-1 cells and rat-1 cells transfected with *myc* oncogene (*myc* cells) showed a low rate of glycolysis that was increased after exposure of the cells to type B transforming growth factor (TGF-β). The uptake of radioactive methylaminoisobutyric acid or L-methionine via system A of amino acid transport also was accelerated after exposure of these cells to TGF-β, with the *myc* cells being most sensitive and the *ras* cells least sensitive. Methionine was found to be a potent inhibitor of glycolysis in *ras* cells as well as in rat-1 or *myc* cells that were exposed to TGF-β. We propose a relationship between the product of the *ras* oncogene (p21) and the protein(s) induced by exposure to TGF-β.**

In a landmark paper (1) it was shown that multiple oncogenes are required to transform a normal rat embryo fibroblast into a tumorigenic cell. It also was shown that an established cell line of rat fibroblasts (rat-1) became tumorigenic after transfection with *ras* oncogene (*ras* cells) but not with *myc* oncogene (*myc* cells). These cell lines are therefore attractive model systems to analyze metabolic changes induced by single oncogenes.

It has been known for many years that most tumors are bioenergetically and metabolically different from normal cells. A high aerobic glycolysis (2) and an increased rate of amino acid uptake via system A (3) are among the most consistent changes, although tumor cells have been described that do not exhibit these metabolic features (cf. ref. 4). It was therefore of interest to explore which of the individual oncogenes are responsible for these metabolic alterations and whether these changes are associated with other biological properties of transformed cells—for example, anchorage-independent growth and growth factor requirements.

We have shown (5, 6) that in Ehrlich ascites tumor (EAT) cells, glycolysis was inhibited 50% by ouabain, a specific inhibitor of the Na^+,K^+-ATPase of the plasma membrane. The activities of glycolytic enzymes were not rate-limiting under these conditions since 2,4-dinitrophenol, an activator of mitochondrial ATPase, stimulated glycolysis in the presence of ouabain >8-fold. This enhanced rate of glycolysis was completely blocked by oligomycin or rutamycin at concentrations that specifically inhibited mitochondrial ATPase. We concluded that the availability of P_i and ADP were rate-limiting for glycolysis.

Glycolysis in some transformed cell lines, however, was not inhibited by ouabain (7). A few were sensitive to oligomycin, suggesting that the mitochondrial ATPase may be activated. Other cell lines were insensitive to both ouabain and oligomycin, but most cell lines were highly sensitive to the bioflavonoid quercetin (7, 8). In many cell lines glycolysis was actually stimulated by oligomycin, as would be expected of cells with highly active mitochondria that compete for ADP and P_i required for glycolysis. Since for each lactic acid formed one ATP is generated, it is essential that during steady-state glycolysis this ATP is broken down to ADP and P_i. The identity of the ATPase responsible for steady-state glycolysis in cells that were not inhibited by either ouabain or rutamycin remained undetermined. Similar conclusions were reached by Jullien *et al.* (8) based on studies of ATP turnover with Rous sarcoma virus-transformed cells. They also observed that quercetin inhibited ATP turnover.

We have observed (9) that normal rat kidney NRK-49F cells exhibited on exposure to type β transforming growth factor (TGF-β) an increase in the rate of glycolysis and in the uptake of methylaminoisobutyrate (MeAIB), a specific substrate of system A of amino acid transport (10). Independently, Inman and Colowick showed that glucose uptake was stimulated by TGF-β in 3T3 cells (11). We also have reported (9) that glycolysis in NRK-49F cells that had been exposed to TGF-β was markedly inhibited by 25 mM methionine, a substrate for system A of amino acid transport, whereas little or no inhibition was noted in the controls that were not exposed to TGF-β. Examination of various established transformed cell lines revealed that in all of them glycolysis was sensitive to methionine, whereas several nontransformed cell lines showed no or moderate inhibition (unpublished data). Although there were differences between cell lines with respect to the time of exposure to methionine (2–16 hr) required to give rise to an inhibition of 50% or more, thus far no exception has been observed among 10 transformed cell lines grown in tissue culture that were tested. However, glycolysis in suspended EAT cells harvested from infected mice was not inhibited by methionine under the conditions tested. Yet, EAT cells grown in tissue culture were among the most sensitive cell lines, showing a significant inhibition after only 30 min exposure to 10 mM methionine. Removal of methionine from the medium resulted in a complete reversal of the inhibition within 2 hr (unpublished data). The capacity to inhibit glycolysis was shared by other substrates transported via system A, including MeAIB, a synthetic compound that is not metabolized.

In view of the fact that methionine inhibits glycolysis in transformed cell lines, we explored the effect of this amino acid and of TGF-β on rat-1 cells and rat-1 variants transfected with *ras* or *myc* oncogenes. We describe in this paper some profound differences in glycolysis, MeAIB uptake, and response to TGF-β that we observed between these cell lines.

MATERIALS AND METHODS

The rat-1, *myc* (R1-CMYC), and *ras* (R1-EJ2) cells were obtained from R. Weinberg. They were grown in Falcon dishes

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Abbreviations: EAT, Ehrlich ascites tumor; *myc* cells, rat-1 cells transfected with *myc* oncogene; *ras* cells, rat-1 cells transfected with *ras* oncogene; NRK, normal rat kidney; TGF, transforming growth factor; PDGF, platelet-derived growth factor; MeAIB, methylaminoisobutyrate.

in Dulbecco's modified Eagle's medium (DME medium) in the presence of 10% Hyclone calf serum. TGF-β was a generous gift from J. Massague; platelet-derived growth factor (PDGF) and the PDGF antibody were from R. Ross. All other materials and methods of lactate and MeAIB transport were as described (9) except that cells were not incubated in assay buffer for 1 hr prior to measuring MeAIB uptake. All cells were grown and exposed to various factors as described in the figure legends. Prior to both assays, the growth medium was removed and the cells were washed with the appropriate assay buffer. For glycolysis, cells were incubated in assay buffer containing 4 mM NaP_i, 20 mM glucose, and factors as indicated and were incubated for 30 min at 37°C. Buffer was removed and assayed for lactate content. For amino acid transport, cells were incubated in assay buffer containing [^{14}C]MeAIB (0.1 mM) for 10 min at 23°C. Cells were thoroughly washed with phosphate-buffered saline and solubilized with 0.2 M NaOH and 1% sodium dodecyl sulfate; radioactive content was measured in a liquid scintillation counter.

RESULTS

Glycolysis in rat-1 and in Cells Transfected with *myc* or *ras*. Cells (rat-1) exposed to TGF-β (250 pg/ml) for 4 hr catalyzed a 2.7-fold faster rate of glycolysis than did cells incubated in the absence of TGF-β (Fig. 1). In the presence of both TGF-β and 25 mM methionine, glycolysis was inhibited 31%, whereas in the absence of TGF-β, the inhibition was only 11%. Quercetin at 8 μg/ml inhibited glycolysis both in the absence and presence of TGF-β. Under the conditions of these experiments (overnight starvation in 0.5% serum), the overall inhibition of glycolysis was moderate but was substantial (>50%) when calculated on the basis of TGF-stimulated glycolysis. When the cells were not starved overnight, the overall inhibition of glycolysis by methionine became much more pronounced, particularly in *ras* cells. It can be seen from Fig. 2 that in rat-1 cells in the presence of TGF-β the inhibition was 28%, in *myc* cells, 41%; and in *ras* cells, 76%. It also can be seen that in *myc* cells the absolute decrease in lactate production by methionine in the presence of TGF-β was greater than in rat-1 cells. This observation is consistent with the increase in MeAIB uptake to be shown later. When cells were not starved overnight and refed the next morning with fresh serum, the methionine inhibition in the presence of TGF-β increased in all cells including rat-1 cells, in line with the observation on the requirement of a serum factor for methionine inhibition documented elsewhere (unpublished data).

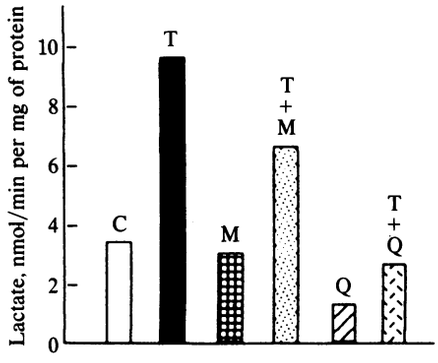

FIG. 1. Effect of TGF-β, methionine, and quercetin on glycolysis in rat-1 cells. Cells were plated at a density of 2.5 × 10^5 cells per 35-mm dish in DME medium containing 10% calf serum. Confluent cells were washed with DME medium and incubated with 2 ml of DME medium containing 0.5% calf serum. After starvation for 16 hr, pure TGF-β (250 pg/ml) and L-methionine (25 mM) were added to the growth medium, the cells were incubated for 6 hr, and lactate production was assayed as described. All factors were present throughout except quercetin (8 μg/ml), which was added just prior to assay. The results represent the mean values of duplicate determinations ± SEM ≤ 15% except for the quercetin control, which has a standard error of 33%. C, control; T, with TGF-β; M, with L-methionine; T + M, with both TGF-β and L-methionine; Q, with quercetin; T + Q, with TGF-β and quercetin.

It also can be seen from Fig. 2 that glycolysis in *ras* cells was >6 times faster than in rat-1 cells. *myc* cells showed only a slightly faster rate of glycolysis (50%) than did rat-1 cells, but this difference was variable and not seen in some experiments. On the other hand, the rate of glycolysis in the presence of TGF-β was consistently higher in *myc* cells than in rat-1 cells. Stimulation of glycolysis by TGF-β in *ras* cells was usually minor (Fig. 2), but the inhibition by methionine in the presence of TGF-β was consistently greater. Consequently and curiously, glycolysis in the presence of both methionine and TGF-β was lower than in the presence of methionine alone. This phenomenon also has been observed in NRK-49F cells (9).

The high aerobic glycolysis in *ras* cells but not in *myc* cells is parallel to the tumorigenicity of these cells: the *ras* cells are tumorigenic, whereas the *myc* cells are not (1). This represents yet another correlation between a high rate of aerobic glycolysis and tumorigenicity.

The effect of the length of incubation with TGF-β on the stimulation of glycolysis is shown in Fig. 3. In the case of rat-1 and *myc* cells, maximal stimulation of glycolysis was achieved within 4–6 hr of incubation with TGF-β. In the case of *ras* cells, glycolysis was much faster (note the different scale) and was hardly affected by the presence of TGF-β. The effect of TGF-β was obliterated by the presence of cycloheximide both in rat-1 and *myc* cells (Fig. 4). In contrast to observations with NRK-49F cells (9), glycolysis in rat-1 and *myc* cells was not significantly affected by cycloheximide, thereby rendering the interpretation of the data less ambiguous.

Transport of MeAIB in rat-1, *myc*, and *ras* Cells. TGF-β stimulated markedly the transport of MeAIB in rat-1 and *myc* cells (Fig. 5). Since the stimulation in *myc* cells was consistently 4- to 6-fold and proportional to the amount of TGF-β added, the uptake of MeAIB is now being used as a routine assay during purification of TGF-β. The rate of MeAIB transport in *myc* cells in the presence of TGF-β was

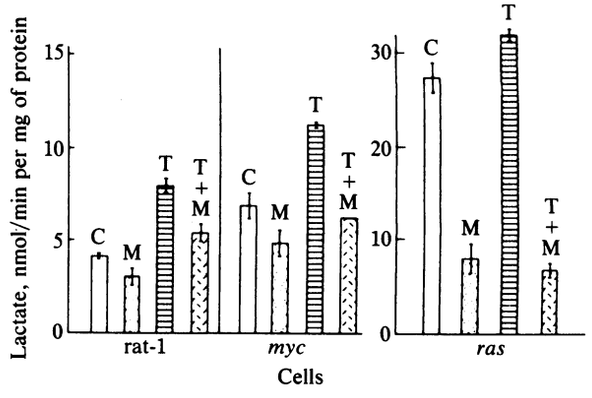

FIG. 2. Effect of TGF-β and methionine on glycolysis in rat-1, *myc*, and *ras* cells. Cells were plated at a density of 1.5–2.0 × 10^5 cells per 35-mm dish in DME medium containing 10% calf serum. After 3 days, TGF-β (200 pg/ml) and L-methionine (25 mM) were added to the growth medium, the cells were incubated for 6 hr, and lactate production was assayed as described. The results represent the mean values of duplicate determinations ± SEM. C, control; M, with L-methionine; T, with TGF-β; T + M, with TGF-β and L-methionine.

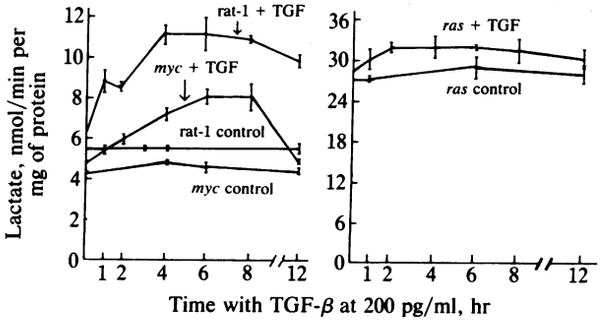

FIG. 3. Time course of TGF-β-stimulated glycolysis in rat-1, *myc*, and *ras* cells. Cells were plated at a density of 2×10^5 cells per 35-mm dish in DME medium containing 10% calf serum. Confluent cells were washed with DME medium and incubated with 2 ml of DME medium containing 0.5% calf serum. After starvation for 16 hr, pure TGF-β (200 pg/ml) was added to the growth medium, the cells were incubated for the times indicated, and lactate production was assayed as described. The results represent the mean values of duplicate determinations ± SEM.

much higher than in *ras* cells, which were again only moderately affected by exposure to TGF-β. In the absence of TGF-β, the uptake of MeAIB was somewhat faster in *ras* cells than in rat-1 or *myc* cells, but this was not observed consistently and varied with the density of the cell culture (12).

In contrast to the stimulation of glycolysis by TGF-β, which required 4–6 hr of incubation, the stimulation of MeAIB uptake in rat-1 and *myc* cells required 8–16 hr (data not shown). This suggests that the stimulation of MeAIB uptake may be secondary to changes induced by the increased glycolysis.

In view of the possible usefulness of the stimulation of MeAIB uptake as an assay for TGF-β, it was necessary to establish how specific the stimulation was for this growth factor. Epidermal growth factor had a small but reproducible stimulatory effect (Fig. 6). PDGF did not stimulate significantly at 1 ng/ml but did at 5 ng/ml. Since both TGF-β and PDGF were isolated from human platelets, it was essential to establish that this stimulation was caused by PDGF and not

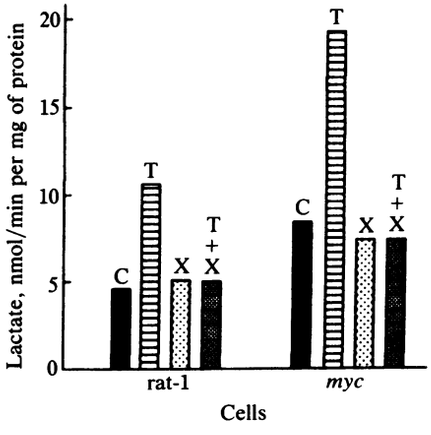

FIG. 4. Cycloheximide sensitivity of TGF-β-stimulated glycolysis in rat-1 and *myc* cells. Cells were plated at a density of 2×10^5 cells per 35-mm dish in DME medium containing 10% calf serum. Confluent cells were washed with DME medium and incubated with 2 ml of DME medium containing 0.5% calf serum. After starvation for 16 hr, pure TGF-β (200 pg/ml) and cycloheximide (1 μg/ml) were added to the growth medium, the cells were incubated 5 hr, and lactate production was assayed as described. The results represent the mean values of duplicate determinations ± SEM ≤ 15%. C, control; T, with TGF-β; X, with cycloheximide; T + X, with both TGF-β and cycloheximide.

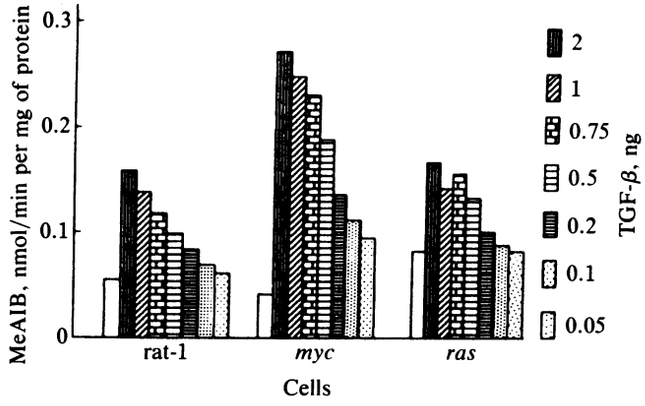

FIG. 5. Concentration-dependent stimulation of MeAIB uptake by TGF-β in rat-1, *myc*, and *ras* cells. Cells were plated at a density of $1.5–2.5 \times 10^5$ cells per 35-mm dish in DME medium containing 10% calf serum and incubated until confluent. Pure TGF-β was added to the growth medium at the concentrations indicated, and the cells were incubated for 16 hr and assayed for [^{14}C]MeAIB uptake as described. The results represent the mean values of duplicate determinations ± SEM ≤ 15%.

by contaminating TGF-β. This was demonstrated by showing that the effect of PDGF, but not that of TGF-β, was eliminated by >80% in the presence of a specific antibody against PDGF (data not shown). None of the other growth factors tested affected significantly the uptake of MeAIB.

DISCUSSION

We have shown in this paper that transfection of rat-1 cells with *myc* or *ras* oncogenes gave rise to distinct differences in patterns of glycolysis and MeAIB uptake and in their response to TGF-β. The *ras* cell had a much higher rate of glycolysis than either rat-1 or *myc* cells. TGF-β stimulated glycolysis in rat-1 and *myc* cells, but even the stimulated rate was considerably lower than that in *ras* cells. Glycolysis in *ras* cells was only slightly stimulated by TGF-β but was highly sensitive to 25 mM methionine. So was glycolysis in rat-1 or *myc* cells that were exposed to TGF-β.

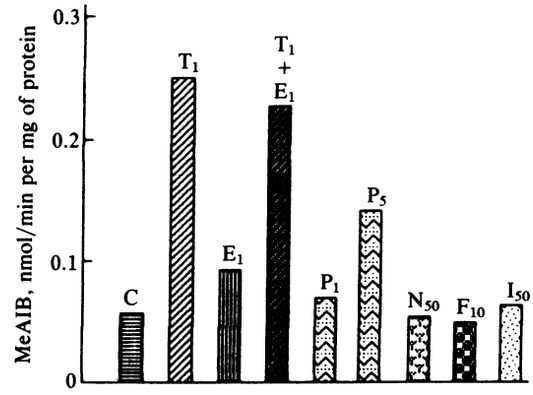

FIG. 6. Comparison of various growth factors on the stimulation of MeAIB uptake in *myc* cells. Cells were plated at a density of 2×10^5 cells per 35-mm dish in DME medium containing 10% calf serum and incubated until confluent. Growth factors were added to the growth medium at the concentrations indicated, and cells were incubated 16 hr and assayed for [^{14}C]MeAIB uptake as described. The results represent the mean values of duplicate determinations ± SEM of ≤ 15%. C, control; T_1, TGF-β at 1 ng/ml; E_1, epidermal growth factor (EGF) at 1 ng/ml; $T_1 + E_1$, TGF-β and EGF, both at 1 ng/ml; P_1, PDGF at 1 ng/ml; P_5, PDGF at 5 ng/ml; N_{50}, nerve growth factor at 50 ng/ml; F_{10}, fibroblast growth factor at 10 ng/ml; I_{50}, insulin at 50 ng/ml.

Why is glycolysis inhibited by methionine? Considering the available data outlined in the introduction, methionine should directly or indirectly inhibit an ATPase. We show elsewhere (unpublished data) that all substrates of system A transport that were tested inhibited the glycolysis of transformed cells, whereas other amino acids did not. Of particular significance is that MeAIB itself is a potent inhibitor of tumor glycolysis since this synthetic substrate for system A is not metabolized by the cell. We tested more recently several synthetic amino acid derivatives for an inhibition of MeAIB uptake. Only three, cycloleucine, methylcysteine, and ethylcysteine, were highly effective, while others were only marginally inhibitory. All three inhibited glycolysis at concentrations that effectively inhibited transport of MeAIB via system A. Therefore, all available data point to a connection between tumor glycolysis and system A transport activity. An obvious possibility is an ATPase attached to this transporter of amino acid uptake. It is well-known that system A transport is inhibited by increasing intracellular concentration of substrate (13). It is of particular interest that this phenomenon referred to as "transinhibition" is more pronounced in hepatoma cells than in normal hepatocytes (14). It seems reasonable to propose that an ATPase associated with an amino acid transporter would be inhibited by accumulating intracellular substrate. Normal human fibroblasts have a much higher intracellular methionine content than do >20 different tumor cells (15). This phenomenon could help to explain the high glycolytic rate of tumors. To lower the glycolytic rate of tumor cells, high external concentration of methionine, the presence of serum, and several hours of incubation are required (unpublished data), perhaps because it is difficult to maintain a high intracellular concentration of this amino acid in tumor cells. This again may be linked to the phenomenon of transinhibition. It is of interest that serum was also shown to be required for a second phenomenon, derepression of system A transport, after amino acid starvation (16), but it is not clear that these observations are necessarily related to the serum requirement for methionine inhibition.

The increased sensitivity of *myc* cells to TGF-β has allowed us to use this system as a rapid biological assay for TGF-β activity. Moreover, the increased response of *myc* to TGF-β points to an alteration in either the TGF-β receptor or in the system A transporter. Since the TGF-β effects were prevented by cycloheximide (9), it seems likely that a newly synthesized protein is required.

The elevated role of glycolysis induced by TGF-β exhibits an increased sensitivity to methionine similar to that of *ras* cells. Could all data be explained by a lower steady-state concentration of methionine in *ras* cells or in TGF-β exposed cells? It has been shown (17) that one of the *ras* gene products present in yeast has a high homology to the G-protein of β-adrenergic receptors. Thus, a possible indirect role of both p21 and TGF-β in the function of the system A transporter can be visualized. In any case, we propose a possible relationship between p21 and the product(s) of TGF-β stimulation. These are questions that are subject to experimental verification.

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