ABSTRACT

The existence and role of an l-arginine:nitric oxide (NO) pathway in human colorectal adenocarcinoma cell lines, SW-480 and SW-620, were investigated. Both cell lines, which derive from the same patient, SW-480 from the primary tumor and SW-620 from its metastatic lesion, were shown to have a cytosolic, Ca2+-independent, NADPH-dependent NO synthase, the activity of which was lower in the cytosol of SW-620. These cells were more potent inducers of platelet aggregation. In contrast, SW-480, which had more NO synthase activity, were less potent inducers of platelet aggregation. Pretreatment of both cell lines with Nω-monomethyl-l-arginine, an inhibitor of NO synthase, potentiated their aggregating effect and made them equally active.

Exogenous l-arginine, NO, and related nitrovasodilators all inhibited platelet aggregation induced by SW-620. The antiaggregating activity of NO was further potentiated by prostacyclin and by M&B22948, a selective inhibitor of cyclic GMP phosphodiesterase. We propose that the generation of NO by tumor cells inversely correlates with their metastatic potential. Furthermore, we show that the lower activity of NO synthase in metastatic cells is due to the presence in these cells of a low molecular weight inhibitor of the NO synthase. In addition, agents which modulate platelet function by a cyclic GMP-dependent mechanism may be useful in the prevention of tumor metastasis.

INTRODUCTION

The capacity of tumors to metastasize is one of the key determinants of their malignant potential. Many studies have implicated platelets in the hematogenous dissemination of tumors (1-8). There is a correlation between the ability of some tumor cells to aggregate platelets in vitro and their propensity for metastasis (2,4,7,8). Platelets form aggregates with tumor cells in the circulation, facilitating their adhesion to vascular endothelium (5). The mechanism of tumor cell-induced platelet aggregation is not clear but seems to vary among tumors of different types. Tumor cells aggregate platelets via thrombin (4), ADP (9), or thromboxane A2 (10) dependent mechanisms and by exposure of platelet receptor glycoproteins Ib and IIb/IIIa (11,12). Other aggregation mechanisms include expression by tumor cells of a trypsin-sensitive protein (13) or of a membrane glycoprotein related immunologically to the platelet IIb/IIIa complex (14). A direct platelet-tumor cell interaction independent of thrombin and of the presence of plasma proteins has also been described (15).

Several pharmacological agents have been investigated as inhibitors of TCIPA. Specific thrombin inhibitors, dansylarginine and hirudin, and apyrase, an enzyme which degrades ADP to AMP and which has no effect on platelet aggregation, all inhibit TCIPA (4,15). Results with the cyclooxygenase inhibitor aspirin have been equivocal (16-18). It has also been suggested that selective inhibitors of thromboxane synthase and/or thromboxane receptor antagonists may be more effective than aspirin in inhibiting TCIPA (19). The pharmacological effectiveness of the former compounds may also depend on the redirection of cyclic endoperoxide metabolism from the proaggregating thromboxane A2 to that of the antiaggregating prostacyclin. Indeed, prostacyclin is the most potent known inhibitor of agonist-induced platelet aggregation as well as of TCIPA (19,21).

Many cells and tissues, notably the vascular endothelium, platelets, and macrophages, synthesize nitric oxide (22-29). This NO is formed from l-arginine (30) by the NO synthase which is inhibited by several l-arginine analogues, including Nω-monomethyl-l-arginine (31-33). Nitric oxide is a potent vasodilator and inhibitor of platelet adhesion and aggregation: these effects are mediated by stimulation of the soluble guanylyl cyclase (34-39).

It has also been demonstrated that cancer cell lines such as murine adenocarcinoma EMT-6 and murine neuroblastoma N1E-115 synthesize NO from l-arginine (40-44). SW-480 and SW-620 are human colorectal adenocarcinoma cell lines which both derive from the same patient (45). SW-480 was isolated from the primary adenocarcinoma arising in the colon, whereas SW-620 was isolated some years later from a lymph node metastasis.

The purpose of the present work was to investigate the existence and the role of the l-arginine:NO pathway in these cancer cell lines in relation to TCIPA. We have also studied the effect of exogenous NO and of related nitrovasodilators on TCIPA induced by SW-620. Finally, we have examined the interactions between prostacyclin and NO as inhibitors of TCIPA.
for 30 min in a Payton dual-channel aggregometer by the method of Born and Cross (47). To determine the effect of endogenous NO on TCIPA, tumor cells were preincubated for 10 min at 37°C alone or with L-NMMA (30 μM) and/or L-arginine (30–100 μM). Cells were then washed, resuspended in Tyrode’s solution, and added to the platelet suspension. In order to investigate the effect of exogenous NO on TCIPA, NO, GTN, or SNAP, either alone or together with prostacyclin or M&B22948, were incubated with platelets 1 min before the addition of SW-620 cells.

In preliminary experiments, it was found that platelet aggregation induced by different concentrations of tumor cells does not correlate with the extent of aggregation, since TCIPA once initiated was maximal and irreversible. However, there was a highly positive relationship (P = 0.9618, n = 3) between TCIPA and lag phase, i.e., the time elapsing from the addition of tumor cells to the occurrence of aggregation. Therefore, lag phase was taken as an inverse index of the aggregating potency of these cells.

Measurement of NO Formation by Cytosols of Tumor Cells. Tumor cells (10^1–10^5 cells), in a total volume of 5 ml of homogenizing buffer (10 mM 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid, 0.32 mM sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% BSA, 0.05% trypsin inhibitor, 10 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor, pH 7.4 at 4°C), were homogenized twice for 3 s with a Soniprep (MSE) homogenizer. The homogenate was centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was passed through a 2-ml column of cation exchange resin (AG 50W-X8) to remove endogenous arginine. In some experiments the effluent was also passed through a 2-ml Sephadex G-25 column to remove low molecular weight components. Nitric oxide synthesis was measured in incubates containing 5 μM oxyhemoglobin and 50% (v/v) tumor cell cytosols in 40 mM potassium phosphate buffer (pH 7.2) containing MgCl2 (1 mM), using a dual wavelength spectrophotometer (Shimadzu UV-3000) as described before (25, 48). Nitric oxide synthesis was initiated by the addition of L-arginine (1–300 μM) and NADPH (300 μM) and it was monitored in the presence or absence of inhibitors of NO synthase. The rate of NO synthesis was linear for 10 min.

Reagents. All reagents for cell culture were from Gibco. Prostacyclin, L-NMMA, SNAP, and L-NO were from Wellcome. L-N-NAME, aspirin, L-arginine, dithiothreitol, soybean trypsin inhibitor, leupeptin, and aprotinin were all obtained from Sigma; GTN was from American Hospital Supplies, and M&B22948 was from May & Baker. Nitric oxide gas (British Oxygen Corp.) was dissolved in helium-deoxygenated water as described previously (23). AG 50W-X8 (Bio-Rad) and Sephadex G-25 (Pharmacia) were obtained as indicated. Human oxyhemoglobin was prepared as described (23).

Statistics. All values are means ± SEM of n experiments. They were analyzed by using analysis of variance and P < 0.05 was considered to be statistically significant.

RESULTS

Effect of L-NMMA on Platelet Aggregation Induced by SW-480 and SW-620. The addition of SW-480 or SW-620 (10^4–3 × 10^6 cells/ml) to the platelets resulted in a cell number-dependent TCIPA as seen by a shortening of the lag phase from 30 to 0 min (Figs. 1 and 2). SW-480 was approximately 10 times less potent than SW-620 in causing TCIPA. Preincubation of these tumor cells with L-NMMA (30 μM) significantly increased the aggregating activity of both cell lines so that a similar dose-response curve was obtained for each in the presence of L-NMMA (Fig. 2). This effect of L-NMMA was completely prevented when l-arginine (100 μM, n = 3) was included in the cell incubate. Preincubation of tumor cells with l-arginine (30 μM) also inhibited platelet aggregation induced by SW-620 (Figs. 1 and 2).

Effect of NO and Other Nitrovasodilators on Platelet Aggregation Induced by SW-620. The addition of SW-620 (10^4 cells/ml) to platelets resulted in platelet aggregation with a lag phase of 5.0 ± 1.6 min (n = 4). Preincubation of platelets with NO, SNAP, or GTN caused a concentration-dependent inhibition of TCIPA, as seen by prolongation of the lag phases (Fig. 3). The order of inhibitory potency was: NO > SNAP > GTN.

Interaction between NO, Prostacyclin, and M&B22948 as Inhibitors of Platelet Aggregation Induced by SW-620. Preincubation of platelets with prostacyclin (1–10 nm) resulted in a concentration-dependent inhibition of SW-620-induced aggregation (10^4 cells/ml; Fig. 4) and a subthreshold concentration of prostacyclin (1 nm) caused a significant potentiation of the antiaggregating activity of NO (0.3 μM; Fig. 4). This action of NO was also potentiated by M&B22948 (1 μM; Fig. 4), which on its own had no significant effect on TCIPA.

Nitric Oxide Synthase Activity in Cytosols of SW-480 and SW-620. The basal rate of NO production by the cytosols was 26 ± 3 and 4 ± 1 pmol/mg protein/min, n = 3, for SW-480 and SW-620, respectively. When depleted of endogenous arginine, they did not generate detectable amounts of NO (<0.01 pmol/mg protein/min; n = 3). The addition of l-arginine (1–300 μM), however, resulted in a concentration-dependent formation of NO (Fig. 5). The amount of NO formed by SW-480...
SW-480 and SW-620 caused a complete restoration of NO synthase activity in cytosol from SW-480 and enhanced NO synthase activity in that from SW-620, so that the amounts of NO formed in these two cytosols were not significantly different from each other (Fig. 6).

In cytosol from SW-480 the formation of NO induced by L-arginine (30 μM) and NADPH (300 μM) was inhibited in a concentration-dependent manner by L-NIO, L-NAME, and L-NMMA (Fig. 7). The order of inhibitory potency was L-NIO > L-NMMA = L-NAME.

**DISCUSSION**

We have demonstrated that the human adenocarcinoma cell lines SW-480 and SW-620 induce aggregation of human washed platelets in vitro. This aggregation was induced in plasma-free medium by aspirin-treated cells. This shows that the presence of plasma proteins, or release of cyclooxygenase was significantly higher than that formed by SW-620 (Fig. 5). When 10% (v/v) of SW-620 cytosol was added to the incubates of SW-480 cells, the rate of NO synthase stimulated by L-arginine (100 μM) and NADPH (300 μM) in SW-480 was inhibited and became not significantly different from that in SW-620 (86 ± 11 and 80 ± 8 pmol/mg protein/min; n = 3).

SW-480 and SW-620 in the presence of L-arginine (100 μM) and NADPH (300 μM) synthesized significantly different amounts of NO; 182 ± 12 and 80 ± 8 pmol/mg protein/min (n = 3), respectively (Fig. 6). The addition of CaCl₂ (200 mM) or ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (1 mM) to these cytosols did not significantly change the amounts of NO formed under these conditions (Fig. 6). However, the NO synthase activity in these cytosols was not detectable once the low molecular weight fraction was removed by passage through a Sephadex G-25 column. The addition of NADPH (300 μM) to the high molecular weight fractions of L-arginine resulted in a concentration-dependent formation of NO by cytosols of SW-480 (○) and SW-620 (■). The amount of NO formed by SW-480 was significantly higher than that by SW-620. The formation of NO was assayed in the presence of NADPH (300 μM). In the absence of L-arginine NO levels were not detectable (<0.01 pmol/mg protein/min). Points, mean of 3 experiments; bars, SEM.
products from tumor cells, are not prerequisites for platelet aggregation induced by SW-480 and SW-620, indicating that its initiation was likely to be a direct platelet-tumor cell interaction (15). Both cell lines originate from the same patient, SW-480 from the primary tumor and SW-620 from a lymph node metastasis. Since SW-620 was a more potent inducer of aggregation than SW-480, our results reinforce the hypothesis (2) that a correlation exists between the ability of tumor cells to aggregate platelets in vitro and their propensity for metastasis.

The platelet aggregating activity of these cell lines was potentiated greatly by pretreatment with L-NMMA, an inhibitor of NO formation in many tissues (31-33), so that they were equally potent platelet-aggregating agents. Furthermore, the aggregating ability was inhibited by the addition of exogenous L-arginine, suggesting the presence of a biologically active L-arginine:NO pathway in both cell lines. This pathway uses two different, NADPH-dependent enzymes to form NO from L-arginine. The first, a constitutive NO synthase is present in cells under physiological conditions, is Ca2+ and calmodulin dependent, synthesizes NO in response to receptor stimulation, and whose expression is inhibited by cycloheximide and glucocorticoids (49-52). This enzyme can be clearly differentiated from an inducible (by a bacterial lipopolysaccharide and by cytokines) NO synthase which is constitutive and Ca2+ independent or whether it is simply an inducible enzyme, expressed in human colorectal adenocarcinoma cells during carcinogenesis in vivo or during cell passage and culture in vitro. The synthesis of NO by SW-480 was inhibited by the NO synthase inhibitors L-NMMA, L-NAME, and L-NIO in the same rank order of potency as seen in platelets and endothelium (26, 33). All these data clearly indicate that NO is produced by human colorectal tumor cell lines during TCIPA in vitro and that its formation down-regulates platelet aggregation.

The differences in the platelet-aggregating potencies of SW-480 and SW-620 cells were abolished in the presence of L-NMMA. Moreover, studies on their respective cytosols showed that the metastasis-derived SW-620 cells formed less NO than the primary tumor-derived SW-480. Furthermore, the addition of as little as 10% of SW-620 cytosol to that of SW-480 was sufficient to inhibit NO synthase activity in the latter. This inhibitory activity could be removed by passing the SW-620 cytosolic fraction through a Sephadex G-25 column, indicating that a low molecular weight component was responsible for this inhibition. To date, this is the first demonstration of the existence of an endogenous inhibitor of NO synthase in tumor cells. The nature and relevance of this factor in the context of NO synthesis in pathological or physiological (if any) conditions remains to be studied. Interestingly, L-arginine decreased the platelet-aggregating properties of SW-620, making them indistinguishable from those of SW-480. These data indicate that in SW-620 L-arginine may be deficient. Thus, both the presence of an endogenous inhibitor of NO synthase and a relative L-arginine deficiency may contribute to the proaggregating properties of SW-620. The differential synthesis of NO by SW-480 and SW-620 cells may have an important significance for the biology of metastasis. However, it remains to be demonstrated whether a deficient capacity to synthesize NO is a general phenomenon characterizing metastatic cells of different origin. The metastatic heterogeneity of subpopulations of cells deriving from the same parent tumor is well recognized (54). Thus, the capacity of tumor cells to synthesize NO may be one of the determinants of this heterogeneity.

![Fig. 6. Characteristics of NO synthase activity in cytosols of SW-480 and SW-620. In the presence of L-arginine (100 μM) and NADPH (300 μM) the amounts of NO produced by SW-480 (C, B) were higher than those produced by SW-620 (C, D). This production was not affected by the presence of calcium (Ca2+, 200 μM) or ethyleneglycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (1 mM). However, when low molecular weight components were removed from the cytosols (HMW) NO formation was abolished. The addition of NADPH resulted in a restoration of NO activity (HMW + NADPH) to similar levels for both cytosols. Columns, mean of 3 experiments; bars, SEM.](image_url)

![Fig. 7. Inhibition of NO synthase in cytosols of SW-480 by L-NIO, L-NMMA, and L-NAME. The formation of NO in cytosols of SW-480 was induced by L-arginine (30 μM) and NADPH (300 μM). This was inhibited in a concentration-dependent manner by L-NIO (○), L-NMMA (△), and L-NAME (■). Points, mean of 3 experiments; bars, SEM.](image_url)
Exogenous NO and the related nitrosodilator SNAP and GTN all inhibited platelet aggregation induced by SW-620 cells, NO being the most potent in this respect. The rank order of potency of the two other nitrosodilators (SNAP > GTN) correlated well with their ability to release NO in vitro (55) as well as with their ability to inhibit agonist-induced platelet aggregation in vitro and ex vivo (56). The inhibitory effect of NO on TCIPA was greatly potentiated by M&B22948, a selective inhibitor of platelet cyclic GMP phosphodiesterase (38), indicating that it is likely to be mediated by an increase in cyclic GMP levels. Moreover, a threshold inhibitory concentration of NO synergized with a subthreshold concentration of prostacyclin to inhibit TCIPA. All these effects, which are similar to that of NO on agonist-induced platelet aggregation (38, 39), suggest that an inhibitory effect of NO and other nitrosodilators on TCIPA in vitro depends mainly on stimulation of cyclic GMP-dependent platelet antiaggregatory mechanisms. However, a direct inhibitory effect of NO on the expression by tumor cells of aggregatory factors cannot be ruled out. The latter effect may be important in vivo where the rheological conditions and interactions between cancer cells and platelets are likely to be modified by the components of the vessel wall as well as by other blood cells.

Thus, the effect of the L-arginine:NO pathway on the biology of tumor cells is likely to be complex. Nitric oxide, the highly reactive effector molecule of this pathway, is a mediator of macrophage-mediated cytotoxicity, and inhibits mitochondrial respiratory function and DNA synthesis in tumor target cells (57–59). Interestingly, some tumor cell lines release large quantities of NO when stimulated by bacterial lipopolysaccharide, γ-interferon, and tumor necrosis factor (40, 41). In addition, these cytokines stimulate the expression of an inducible NO synthase in other cells, such as the vascular endothelium (50). Thus, NO released from macrophages, endothelium, and tumor cells themselves in response to cytokines can exert an antitumor cytotoxic effect on the target cells. In addition to a tumoricidal action, the generation of NO during TCIPA and its inhibitory effect on this aggregation may reduce the metastasizing ability of tumor cells. However, the obvious benefits from the actions of NO may be outweighed by the side effects resulting from NO-induced hypotension, this being one of the most severe manifestations of septic shock (60).

Our results have a clinical significance in that they demonstrate that nitrosodilators, given alone or in combination with prostacyclin or phosphodiesterase inhibitors, can effectively arrest TCIPA. They also cast a new light on the proposal that dietary arginine is a good adjunct to the therapy of the malignant disease (for references, see Ref. 61). Finally, the generation of NO by tumor cells may be a useful laboratory marker of their metastatic potential.

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Human Colorectal Adenocarcinoma Cells: Differential Nitric Oxide Synthesis Determines Their Ability to Aggregate Platelets

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