Role of urokinase and its receptor in basal and stimulated colonic epithelial cell migration in vitro

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Abstract

Background—Migration of colonic epithelial cells is important for mucosal repair following injury. The urokinase (u-PA) system regulates migration in other cell types.

Aim—To examine the role of u-PA and its receptor (u-PAR) in colonic epithelial cell migration.

Methods—Migration was assessed over 24 hours in circular wounds made in confluent monolayers of LIM1215 and Caco-2 human colon cancer cells. The function of u-PA and u-PAR was ablated with antisense oligonucleotides to block expression, with synthetic u-PA peptides to block interaction, and with aprotinin to block u-PA mediated proteolysis.

Results—Migration was stimulated two to threefold by exogenous u-PA, an effect dependent on u-PAR binding but independent of u-PA mediated mitogenesis and proteolysis. Expression of u-PA and u-PAR was inhibited by 80% by the appropriate antisense oligonucleotide. Basal migration and the mitogenic effects of butyrate, epidermal growth factor, and phorbol-12-myristate-13-acetate were suppressed by the u-PAR antisense oligonucleotide (40–60%) but were at best minimally affected following inhibition of u-PA expression and binding.

Conclusions—In an in vitro model of wounded colonic epithelium, u-PAR promotes cell migration through mechanisms that are not exclusively dependent on u-PA binding. Therefore, u-PA and u-PAR may contribute to colonic mucosal repair in vivo. (Gut 2000;47:105–111)

Keywords: colon; migration; urokinase; urokinase receptor; epidermal growth factor; butyrate; protein kinase C

The urokinase system comprises several components. Urokinase-type plasminogen activator (u-PA) is secreted as a single chain proenzyme form and converted by proteolytic cleavage to an enzymatically active two chain form.¹ The receptor for u-PA (u-PAR) is a 55–65 kDa glycosylphosphatidylinositol-linked protein that binds the epidermal growth factor-(EGF)-like domain (amino acids 20–32) of two chain u-PA.² Plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2) inhibit u-PA function by forming a u-PA/PAI/u-PAR complex which results in the degradation of u-PA/PAI and recycling of u-PAR.¹ A well characterised function of the urokinase system is localised pericellular generation of plasmin through

receptor bound u-PA mediated catalysis of plasminogen.¹ Plasmin is implicated in the proteolysis of cell/cell contacts, cell/matrix contacts, matrix components, and in the activation of latent matrix bound growth factors.^{3–4} The catalytic activity of u-PA is known to regulate the metastasis of cancer cells (reviewed by Andreason and colleagues⁵) and migration of normal corneal epithelial cells over their native matrix in vitro.⁶

Migration of a range of cell types over an artificial substratum in vitro is dependent on the binding of u-PA but not on its proteolytic activity.^{7–10} Several studies have indicated that u-PA binding activates several signalling molecules implicated in the control of cell migration, such as focal adhesion kinase,¹¹ protein kinase C (PKC),⁸ extracellular signal regulated kinase,^{11–12} Janus associated kinases,^{13–14} src family tyrosine kinases,¹⁵ and G proteins.¹⁶ Although u-PAR lacks a transmembrane and intracellular domain and, therefore, intrinsic signal transduction potential, its ability to associate with cytoskeletal components and/or integrins at sites of focal contact^{3–8–17–20} is a likely explanation for this phenomenon.

There is also evidence which indicates that components of the urokinase system exert cellular effects independent of u-PA binding. For example, u-PAR regulates cellular adhesion and migration by modifying the adhesive properties of integrins,²⁰ and by binding to the extracellular matrix component, vitronectin.²¹ Furthermore, the latter appears to be controlled by PAI-1.¹⁰ That the urokinase system plays a complex role in the movement of cells in vitro is supported by the demonstration that selective suppression of the expression of u-PAR, but not u-PA, abolishes monocyte chemotaxis.²²

At present, there is no published evidence on the role of the urokinase system in the migration of normal colonic epithelial cells in response to mucosal wounding (termed restitution) or in the migration elicited by wounding of colonic epithelial cell monolayers in vitro. A role is likely, given the importance of u-PA and u-PAR in the migration of other cell types and expression of u-PA by colonic epithelial cells in the surface compartment.²³ A range of colon cancer cell lines are known to express u-PA and u-PAR mRNA and protein.^{24–26} Furthermore, several colon cancer cell motogens identified in this laboratory, such as the short chain fatty acid, butyrate, ligands for the EGF receptor,^{27–28} and

Abbreviations used in this paper: EGF, epidermal growth factor; MD, migration differential; PKC, protein kinase C; ON, oligonucleotide; PMA, phorbol-12-myristate-13-acetate; u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; LDH, lactate dehydrogenase; PKC, protein kinase C.

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the activator of PKC, phorbol-12-myristate-13-acetate (PMA),²⁸ modulate mRNA and/or protein expression of one or both in a range of cell types.^{24–26 29–33}

The aim of our study was to determine the effect of exogenous u-PA on cell migration in an *in vitro* model of wounded colonic epithelium, to identify mechanisms by which it acts, and to examine the role of u-PA and u-PAR in basal migration and when stimulated by butyrate, EGF, and PMA.

Materials and methods

CELL CULTURE

The human colon cancer cell lines used were the moderately differentiated LIM1215 (passage 20–25) (a kind gift from Dr Robert Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia) and the well differentiated Caco-2 (passage 25–35) (American Type Culture Collection (ATCC) HTB 37). The materials used for cell culture were as previously described.²⁷

WOUNDING ASSAY

Cell migration was assessed in circular wounds created in confluent cell monolayers by a rotating Teflon tip,²⁷ a method modified from that of Watanabe and colleagues.³⁴ The cell free area of the wounds was calculated as previously described.²⁷ Migration over 24 hours was expressed as the migration differential—the mean change in cell free area of 10 replicate wounds.

FACTORS USED TO MODULATE MIGRATION

Human recombinant two chain u-PA (No 125) was obtained from American Diagnostica (Epping, New South Wales, Australia). Known motogens used were: the sodium salt of butyrate (Sigma-Aldrich, St Louis, Missouri, USA) dissolved in PBS to form a sterile solution, pH 7.4; EGF (Auspep, Parkville, Victoria, Australia); and the phorbol ester, PMA (Sigma-Aldrich). All migration experiments were conducted using serum free medium supplemented with 1 mg/ml BSA (Sigma-Aldrich), unless otherwise noted.

Expression of u-PA and u-PAR was inhibited by 19mer antisense oligonucleotides (ONs), 5'-GCGCCAGCAGGGCTCTCAT-3' and 5'-TCTTCAGAGGAGCATCCAT-3' respectively (Geneworks, Adelaide, South Australia, Australia), which correspond to positions 426–444 of u-PA cDNA³⁵ and 720–738 of u-PAR cDNA,³⁶ respectively. These sequences inhibit their expression in human monocytes.²² Two phosphorothioate substitutions were added at either end to prevent nuclease digestion. Similarly modified sense ONs were used as negative controls. The ONs were complexed with the N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP) liposomal transfection reagent (Boehringer Mannheim, Castle Hill, New South Wales, Australia) according to the manufacturer's instructions and applied to preconfluent cells for 24 or 48 hours at final concentrations of 5–20 μ M (ONs) and 10 μ M (DOTAP). After incubation, the mixture was

removed, fresh medium added, and experiments performed in the absence of ONs.

Two synthetic u-PA peptides constructed by Dr Hye-Yeong Min (Chiron Technologies, San Francisco, California, USA), one corresponding to amino acids 1–48 of human u-PA (u-PA 1–48) and a complex containing two u-PA 1–48 sequences conjugated with two human Fc portions (u-PA 1–48/Ig), were used to compete with endogenous u-PA binding. The protease inhibitor aprotinin (Bayer Australia, Pymble, New South Wales, Australia) was used which blocks u-PA proteolysis mediated corneal epithelial cell migration.⁶ Aprotinin concentrations are expressed in kallikrein inhibitor units (KIU). All experiments using aprotinin were conducted in culture medium containing 10% fetal calf serum to ensure the presence of plasminogen. All factors were applied for two hours before and throughout the migration experiments. The inhibitor of DNA synthesis, mitomycin C, was obtained from Crown Scientific (Burwood, Victoria, Australia) and applied to wounded monolayers for two hours before the addition of u-PA.

BIOCHEMICAL ASSAYS

Cell supernatants and homogenates were collected and stored as previously described.²⁷ The protein content of cell homogenates was measured by the method of Bradford.³⁷ Levels of u-PAR in cell homogenates (No 394, American Diagnostica), and u-PA in cell homogenates and culture supernatants (No 393, American Diagnostica), were measured using commercially available ELISA kits. Values are expressed as ng/mg protein. Cell associated u-PA activity was assayed using the method of Coleman and Green,³⁸ and values expressed as mIU/mg protein. All experiments were conducted in wounded monolayers identical to those used in the migration experiments, unless stated otherwise.

STUDIES OF CELL TURNOVER AND VIABILITY

³H thymidine uptake, adherent cell number, and trypan blue exclusion were determined in wounded LIM1215 monolayers as previously described.²⁷ The cells were pulsed with ³H thymidine from 16 to 24 hours after wounding and uptake in DNA was expressed as disintegrations per minute (dpm) relative to cell number counted in parallel wells. Release of the cytosolic enzyme lactate dehydrogenase (LDH) into supernatants of wounded LIM1215 monolayers was assayed as previously described²⁷ and values expressed as U/mg protein. All experiments were conducted in wounded monolayers identical to those used in the migration experiments.

STATISTICAL EVALUATION

Group data from all experiments are expressed as mean (SEM). Experimental and control groups were compared using a two tailed paired *t* test. A *p* value ≤ 0.05 was considered statistically significant. All statistical analyses were performed on raw data using Minitab release 8 (Minitab Inc., State College, Pennsylvania, USA, 1991).

Figure 1 (A) Effects of increasing concentrations of u-PA on the migration of LIM1215 and Caco-2 cells after 24 hours. Values are mean (SEM) of four experiments. *Lowest concentration at which stimulation of migration was observed ($p<0.05$, paired t test). (B) Effect of two hours pretreatment with the DNA synthesis inhibitor mitomycin C (20 μ g/ml) on the stimulatory effect of u-PA 100 ng/ml on migration and ^{3} H thymidine uptake measured in the same wounded LIM1215 monolayers. Values are mean (SEM) of four experiments (** $p<0.01$ relative to stimulatory effect alone; paired t test).

Results

EFFECTS OF u-PA ON CELL MIGRATION AND PROLIFERATION

As shown in fig 1A, exogenous u-PA stimulated the migration of LIM1215 and Caco-2 cells in a concentration dependent manner. Optimal effects in both cell lines were obtained at a u-PA concentration of 100 ng/ml. This concentration resulted in motogenic effects of approximately three and twofold stimulation, respectively. In both cell lines there were only minimal stimulatory actions on migration associated with u-PA concentrations at or below 10 ng/ml.

The motogenic effect of u-PA was unaffected by a range of concentrations (100–500 KIU) of the protease inhibitor aprotinin (data not shown). In four experiments using 500 KIU aprotinin,

there was only 3 (4)% ($p=0.32$ relative to stimulatory effect alone) and 5 (2)% ($p=0.18$) inhibition of u-PA-stimulated migration in LIM1215 and Caco-2 cells, respectively. This suggests that generation of plasmin was not required for u-PA mediated motogenesis particularly as the motogenic effects of u-PA described above were obtained in serum free medium (presumably containing no plasminogen).

In LIM1215 cells, u-PA stimulated ^{3} H thymidine uptake in a concentration dependent manner. Compared with control levels in four experiments (11 940 (865) dpm/ 10^5 cells), 5 ng/ml u-PA had no significant effect (10 581 (756) dpm/ 10^5 cells; $p=0.14$) whereas 20 ng/ml (14 630 (1329) dpm/ 10^5 cells; $p<0.03$) and 100 ng/ml (19 564 (1919) dpm/ 10^5 cells; $p<0.01$) increased the rate of DNA synthesis by 23 (5)% and 65 (8)%, respectively. A similar pattern of effect was observed for the action of u-PA on adherent cell number (data not shown). However, studies with the DNA synthesis inhibitor mitomycin C indicated that increased cell number within the monolayer was unlikely to be a mechanism of u-PA enhanced wound closure. As shown in fig 1B, mitomycin C (20 μ g/ml) suppressed the stimulatory effect of 100 ng/ml u-PA on ^{3} H thymidine uptake by 89 (9)% ($p<0.01$ relative to mitogenic effect alone) but did not inhibit u-PA mediated motogenesis ($p=0.26$ relative to motogenic effect alone) in the same monolayers. Consistent with previous observations, mitomycin C inhibited ^{3} H thymidine uptake by approximately 90% and induced modest (10%) stimulation of migration.²⁷

EXPRESSION OF u-PA AND u-PAR BY LIM1215 AND Caco-2 CELLS

Both LIM1215 and Caco-2 cells constitutively expressed u-PA and u-PAR. Levels of supernatant and cell associated u-PA, cell associated u-PAR, and cell associated u-PA activity, which measures the catalytic activity of receptor bound u-PA, under basal conditions are shown in table 1. The total amount of u-PA produced by LIM1215 cells corresponded to a concentration of approximately 3 ng/ml, while Caco-2 cells produced considerably less (<1 ng/ml).

The modulating effects of factors previously shown to potently stimulate the migration of LIM1215 and Caco-2 cells (butyrate (2 mM), EGF (20 ng/ml), and PMA (10 nM)^{27 28}) were also investigated. Their effects on the indices listed above after a 24 hour incubation are

Table 1 Indices of the urokinase system under basal and stimulated conditions

	Super u-PA (ng/mg protein)			Cellular u-PA (ng/mg protein)			Cellular u-PAR (ng/mg protein)			Cellular u-PA activity (mIU/mg protein)		
	Level	% Change		Level	% Change		Level	% Change		Level	% Change	
LIM1215												
Basal	0.71 (0.05)	—		0.69 (0.11)	—		1.01 (0.2)	—		479 (45)	—	
Butyrate	0.46 (0.03)	–30 (5) ^a		0.52 (0.11)	–35 (4) ^a		3.78 (0.73)	289 (74) ^c		97 (9)	–79 (2) ^d	
EGF	2.17 (0.16)	214 (38) ^a		NT ^b	NT		3.89 (0.21)	334 (54) ^a		925 (80)	189 (24) ^a	
PMA	2.33 (0.25)	267 (60) ^a		NT	NT		4.74 (0.31)	425 (57) ^a		990 (99)	245 (67) ^a	
Caco-2												
Basal	0.2 (0.06)	—		0.05 (0.02)	—		0.15 (0.02)	—		184 (12)	—	
Butyrate	0.35 (0.07)	68 (6) ^a		0.13 (0.01)	172 (38) ^c		0.35 (0.04)	137 (17) ^d		417 (42)	125 (17) ^d	

Supernatant (Super) u-PA, expression of cellular u-PA and u-PAR, and cell associated u-PA activity were assayed in wounded LIM1215 and Caco-2 monolayers after a 24 hour incubation with and without exposure to 2 mM butyrate, 20 ng/ml EGF and 10 nM PMA. Values are mean (SEM) of 3–6 experiments. ^a $p<0.05$; ^bnot tested; ^c $p<0.01$; ^d $p<0.001$. All p values are expressed relative to basal levels and were determined using a paired t test.

Table 2 Characterisation of the u-PA and u-PAR oligonucleotides (ONs)

	Supernatant u-PA		Cellular u-PAR		u-PA stim. migration		u-PA stim. ³ H thy. uptake
	LIM1215	Caco-2	LIM1215	Caco-2	LIM1215	Caco-2	LIM1215
u-PA antisense	85 (7) ^{a,b}	82 ^d	NT	NT	NT	NT	NT
u-PA sense	3 (4) ^a	5 ^d	NT	NT	NT	NT	NT
u-PAR antisense		NT ^c	82 (5) ^{a,b}	78 ^d	77 (8) ^c	68 (12) ^{cd}	71 (13) ^{ef}
u-PAR sense		NT	2 (6) ^a	4 ^d	5 (2) ^c	1 (6) ^c	8 (5) ^e

Effects of 48 hour exposure to u-PA and/or u-PAR antisense and sense ONs (20 mM) on supernatant u-PA levels, cellular u-PAR expression, and 100 ng/ml u-PA stimulated (stim.) migration and ³H thymidine (thy.) uptake in wounded LIM1215 and Caco-2 monolayers. Values are the results of 2–4 experiments. ^aPercentage inhibition (mean (SEM)) compared with controls; ^bp<0.01 relative to control, paired *t* test; ^cnot tested; ^dpercentage inhibition (mean of two experiments) compared with controls; ^epercentage inhibition (mean (SEM)) of stimulatory effect; ^fp<0.01 relative to stimulatory effect alone, paired *t* test.

shown in table 1. Butyrate induced an approximately twofold increase in total u-PA in Caco-2 cells but markedly reduced total u-PA in LIM1215 cells. Both EGF and PMA stimulated supernatant u-PA levels in LIM1215 cells by threefold or higher. Effects on cell associated u-PA activity paralleled those on u-PA levels in both cell lines and for all factors examined. Expression of u-PAR was increased by 2–5-fold by all factors tested in both cell lines.

CHARACTERISATION OF u-PA AND u-PAR ONs
The effects of 48 hour exposure to the u-PA and u-PAR antisense ONs (20 μM) on basal supernatant u-PA levels and cell associated u-PAR expression in wounded LIM1215 and Caco-2 monolayers are shown in table 2. Such treatment was found to be optimal in preliminary concentration and time course experiments (data not shown). Levels of u-PA and u-PAR protein were reduced by approximately 80% by the appropriate antisense ON in both cell lines. The suppressive actions of the antisense ONs on protein expression were selective as the corresponding sense ONs had only minimal effects. The antisense ONs also reduced the stimulatory effect of EGF, PMA, and butyrate on u-PAR and/or u-PA levels in LIM1215 and Caco-2 cells by 70–85% (data not shown).

The efficacy of the u-PAR antisense ON in reducing functional levels of u-PAR was confirmed in migration and ³H thymidine uptake studies (table 2). The stimulatory effects of 100 ng/ml u-PA on these indices in wounded LIM1215 monolayers were inhibited by 70–80% by the antisense ON while the sense ON was ineffective. There was a similar pattern of inhibition of u-PA mediated motogenesis in Caco-2 cells. Assessment by light microscopy indicated that the u-PAR antisense ON also induced morphological changes in subconfluent LIM1215 cells. These treated cells were larger and more rounded than untreated cells which had a spindle shaped morphology. The corresponding sense ON, and also the u-PA antisense ON, had no such effects.

ROLE OF ENDOGENOUS u-PAR AND u-PA IN PROLIFERATION AND MIGRATION
Basal ³H thymidine uptake
Basal ³H thymidine uptake in wounded LIM1215 monolayers (13 899 (3122) dpm/10⁵ cells) was not altered by the u-PAR antisense (13 135 (978) dpm/10⁵ cells; p=0.16 relative to control) or u-PA antisense (11 128 (2876) dpm/10⁵ cells; p=0.08) ON in three experiments. A similar pattern of effect was observed in adherent cell counts (data not shown).

Basal migration
Three lines of evidence suggest that u-PA plays a minor role in basal migration. Firstly, the u-PA antisense ON had no effects on basal migration in either LIM1215 (p=0.18) or Caco-2 (p=0.19) cells (figs 2, 3). Secondly, the effect of competitively blocking the interaction of u-PA and u-PAR with synthetic u-PA peptides was investigated. As these peptides lack a catalytic domain, their efficacy in inhibiting endogenous u-PA binding in LIM1215 cells was assayed by the extent to which they reduced basal cell associated u-PA activity after 24 hours. Both peptides induced a concentration dependent reduction in u-PA activity with optimal inhibitory effects of u-PA 1–48 and u-PA 1–48/Ig at concentrations of 100 nM and 50 nM, respectively (fig 4). However, neither peptide had any inhibitory effect on basal migration in Caco-2 (fig 3) or LIM1215 (fig 5A, B) cells. Thirdly, supernatant u-PA levels were not increased 24 hours after the wounding of either LIM1215 (6 (5)% above control, p=0.09, n=3) or Caco-2 monolayers (11 (7)% below control, p=0.07, n=6).
The u-PAR antisense ON inhibited basal migration of LIM1215 (p<0.01) and Caco-2 (p<0.02) cells by more than 40% while the u-PAR sense ON had no effect in either cell line (p=0.32 and 0.19, respectively) (figs 2, 3). These results are apparently consistent with the fact that expression of u-PAR in wounded LIM1215 and Caco-2 monolayers after 24 hours was enhanced by 32 (9)% (p<0.02, n=5) and 26 (9)% (p<0.04, n=6), respectively, compared with unwounded monolayers. The reduction in basal wound closure induced by the

Figure 2 Effects of 48 hour exposure to u-PAR and u-PA antisense and sense ONs (20 μM) on basal LIM1215 migration and the motogenic effects of EGF (20 ng/ml), butyrate (2 mM), and PMA (10 nM). Values are mean (SEM) of four experiments and represent percentage inhibition of basal and stimulated migration (**p<0.01 relative to control; †p<0.05, ††p<0.01 relative to stimulatory effect alone; all paired *t* test).

Figure 3 Effect of 48 hour exposure to u-PAR and u-PA antisense and sense ONs (20 μ M), and the synthetic u-PA peptides u-PA 1-48 (100 nM) and u-PA 1-48/Ig (50 nM) on basal Caco-2 migration and the motogenic effect of butyrate (2 mM). Values are mean (SEM) of three experiments and represent percentage inhibition of basal and butyrate stimulated migration (* p <0.05 relative to control; † p <0.05 relative to stimulatory effect alone; all paired t test).

Figure 4 Effect of increasing concentrations of the synthetic u-PA peptides u-PA 1-48 and u-PA 1-48/Ig on basal cell associated u-PA activity in wounded LIM1215 monolayers after 24 hours. Values are mean (SEM) of three experiments and are expressed as percentage of basal activity (* p <0.05 relative to control; paired t test).

u-PAR antisense ON is unlikely to have resulted from toxicity as the treated LIM1215 cells had similar LDH release as untreated cells (398 (34) U/mg compared with 374 (46) U/mg; p =0.22, n =3), >95% viability in trypan blue exclusion experiments, and remained adherent to the substratum.

Butyrate stimulated migration

In LIM1215 cells, the motogenic effect of 2 mM butyrate was not inhibited by the u-PA antisense ON (fig 2) or by u-PA peptides (fig 5A, B). In contrast, the motogenic effect of butyrate in Caco-2 cells was modestly inhibited (15–20%) by the u-PA antisense ON (p <0.03 relative to stimulatory effect alone) and 50 nM u-PA 1-48/Ig (p <0.03) while 100 nM u-PA 1-48 exerted a similar effect that just failed to reach statistical significance (p =0.058) (fig 3).

The motogenic effect of butyrate was markedly inhibited (50–60%) by the u-PAR antisense ON in both LIM1215 cells (p <0.01 relative to stimulatory effect alone) and Caco-2 cells (p <0.02) while the corresponding sense ON had no effect (figs 2, 3). Suppression of butyrate stimulated wound closure by the u-PAR antisense ON was not due to toxicity as LDH release into LIM1215 cell supernatants by ON treated cells subsequently exposed to butyrate (453 (62) U/mg) was not different

Figure 5 Effect of increasing concentrations of the synthetic u-PA peptides u-PA 1-48 (A) and u-PA 1-48/Ig (B) on basal and butyrate stimulated LIM1215 migration after 24 hours. Values show the migration differential and are mean (SEM) of three experiments. Neither peptide had any significant effect on either index at any concentration.

(p =0.24) to that in cells treated with butyrate alone (432 (56) U/mg) in three experiments.

EGF and PMA stimulated migration

IN LIM1215 cells, the u-PA antisense ON induced mild inhibition (<20%) of the motogenic effect of 20 ng/ml EGF (p <0.03) and tended to reduce that of 10 nM PMA, although this effect just failed to reach statistical significance (p =0.054) (fig 2). The motogenic effects of EGF and PMA were inhibited by 40–50% by the u-PAR antisense ON (p <0.01 and 0.02 relative to stimulatory effect alone, respectively) (fig 2). Abrogation of stimulated wound closure induced by the u-PAR antisense ON was not due to toxic effects as in three experiments the combination of PMA or EGF and the ON (471 (71) U/mg and 379 (43) U/mg, respectively) did not induce significantly greater LDH release than that of PMA (453 (51) U/mg) or EGF (368 (23) U/mg) alone (p =0.17 and 0.35, respectively).

Discussion

Migration and proliferation of the human colon cancer cell lines LIM1215 and Caco-2 were stimulated in a concentration dependent manner by u-PA. Maximal effects were obtained at supraphysiological concentrations. Selective suppression of u-PAR expression markedly ablated u-PA mediated motogenesis and mitogenesis, indicating that these processes were mediated by binding to functional u-PAR. A wide range of signalling molecules activated through u-PA binding have been implicated in regulating the resultant cellular migration and/or

proliferation.^{8 11-16} Those responsible for the effects described in the present study are unknown and their identification will be the focus of further investigation. This is the first report of such effects of u-PA in gastrointestinal epithelial cells although similar actions have been described in other cell types.^{7-10 39 40}

Despite the fact that u-PA stimulated indices of proliferation in LIM1215 cells, its stimulatory action on wound closure was not ablated in monolayers where u-PA mediated mitogenesis was markedly inhibited. It has been previously shown in this laboratory and by others that the mitogenic effects of other mitogenic peptides, such as EGF, are largely independent of increased cell number within the monolayer.^{27 28 41} The mitogenic effect of u-PA was also mediated independently of another well characterised process initiated through its binding to u-PAR, generation of plasmin.¹ Other groups have also reported an apparent segregation between mitogenesis and proteolysis in cells grown on plastic, as occurred in the present study, or purified matrix components *in vitro*.⁷⁻¹⁰ In contrast, migration of corneal epithelial cells requires u-PA proteolytic activity when native matrix is used as the substratum.⁶ Therefore, the role of u-PA mediated proteolysis in colonic epithelial cell migration needs to be defined in further studies using a more physiological substratum.

Neither the selective ablation of constitutive u-PA expression nor partial suppression of u-PA binding inhibited basal proliferation and/or migration of LIM1215 and Caco-2 cells. The minimal influence of endogenous u-PA was most likely due to its substantially lower production by the cells compared with the lowest concentration of exogenous u-PA required to exert a clear stimulatory effect. In contrast, basal migration of the cells was inhibited when u-PAR expression was selectively ablated. This was associated with a change in cellular morphology from the characteristic spindle shape of the cells to a more rounded phenotype, which may be a reflection of the interaction of u-PAR with cytoskeletal components.^{8 19} A similar obligate role for u-PAR has previously been found in the chemotactic migration of human monocytes.²²

Wounding of LIM1215 and Caco-2 cell monolayers was associated with upregulated expression of cellular u-PAR but not secretion of u-PA into cell supernatants. This contrasts with findings in several other cell types where increases in both u-PA and u-PAR at the leading edge of migrating bovine endothelial cells,^{42 43} corneal epithelial cells,⁶ keratinocytes,⁴⁴ and smooth muscle cells¹⁸ are observed. However, it is possible that this apparent discrepancy could be related to the methodology used to measure u-PA expression. Measuring protein expression in cell supernatants may not be as sensitive at detecting changes localised to cells adjacent to the wound as are, for example, zymographic assays for u-PA.⁴²

Stimulation of endogenous u-PA expression and binding by butyrate in Caco-2 cells, and EGF and PMA in LIM1215 cells, appeared to contribute only minimally to the mechanism(s)

by which they stimulated migration. It is likely that the levels of u-PA by these factors were only sufficient to weakly affect the signalling pathways responsible for motogenesis following u-PAR binding. Ablation of u-PA expression and binding failed to suppress the mitogenic effect of butyrate in LIM1215 cells, which appears consistent with its observed reduction of u-PA expression and cell associated activity. Similar effects of butyrate on u-PA mRNA and protein expression in colon cancer cell lines have been reported.²⁴⁻²⁶

The mitogenic effects of butyrate in LIM1215 and Caco-2 cells, and EGF and PMA in LIM1215 cells, were partly dependent on the stimulated expression of u-PAR. Therefore, obligate expression of u-PAR played a more important role than u-PA binding in the mitogenic effect of three different factors in two different cell lines. Such an effect of butyrate on u-PAR expression is contrary to other studies in colon cancer cell lines where it reduces steady state levels of u-PAR mRNA.²⁴⁻²⁶ However, butyrate was used at higher concentrations than in the present study, and at levels where evidence of toxicity was found.^{27 45 46}

Our study indicated that there were apparently both u-PA dependent and independent mechanisms by which u-PAR regulated migration following wounding of LIM1215 and Caco-2 monolayers. Whether this would also apply if the cells were undergoing a different form of migration, such as in chemotaxis assays, is worthy of further investigation. u-PAR is known to exert several cellular effects largely independent of u-PA binding. For example, it mediates the transduction of mechanical forces across the cell membrane⁴⁷ which may be involved in initiating migration following mechanical wounding of cell monolayers, and modulates cell adhesion by binding to vitronectin²¹ or to activated integrins, thereby regulating their adhesive characteristics.²⁰ Furthermore, a study in a monocytic cell line showed that proteolytic cleavage of u-PAR results in a soluble form of u-PAR that associates with the cell membrane and transmits similar signals as u-PA binding.¹⁵ Therefore, in addition to its role as a binding protein for u-PA, u-PAR may also influence migration through regulation of cellular adhesion to the substratum and/or activation of signalling pathways involved in its initiation and propagation, independent of u-PA.

Diseases characterised by mucosal ulceration, such as inflammatory bowel disease, ulcerative colitis, and post-chemotherapy mucositis, are of considerable clinical concern. The identification of physiologically relevant factors that control the repair of these ulcers, particularly the early restitutive phase characterised by epithelial migration, may potentially lead to the development of novel therapeutic approaches that hasten repair of, or limit damage to, the mucosa. Clearly, the role of u-PA and u-PAR in colonic epithelial cell migration needs to be established in models using normal epithelium, such as colonic mucosal explants, isolated colonic

mucosa mounted in Ussing chambers, and marsupialised colonic loops in animals.

In conclusion, migration in the human colon cancer cell lines, LIM1215 and Caco-2, following monolayer wounding is markedly stimulated by exogenous u-PA. This motogenic effect required binding to functional u-PAR but was not dependent on the subsequent mitogenic or proteolytic activity of u-PA. Despite its constitutive secretion, endogenous u-PA had a minor role in regulating basal migration and that stimulated by butyrate, EGF, and PKC activation compared with u-PAR in both cell lines. Therefore, u-PAR appears to promote migration through mechanisms that are not exclusively dependent on u-PA binding. As u-PA and u-PAR are expressed by normal colonic epithelium, especially in the differentiated compartment, the results of this study suggest that the urokinase system may be involved in the regulation of migration of normal colonic epithelial cells in response to mucosal wounding.

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