Original article

Mechanisms of endothelin 1-stimulated proliferation in colorectal cancer cell lines

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Background: **The peptide endothelin (ET) 1 promotes proliferation in a number of epithelial cancers. The aim of this study was to identify the mechanism of ET-1-stimulated proliferation in colorectal cancer cells *in vitro*.**

Methods: **The effects of ET-1 on colorectal cancer cell lines HT29, LIM1215 and SW620 were studied. Cells were cultured with ET-1 plus antagonists/inhibitors to ET_A or ET_B receptors, G protein subtypes, phosphoinositide 3-kinase (PI3K) or protein kinase C (PKC). DNA replication and apoptosis were investigated by 5-bromo-2′-deoxyuridine incorporation and Annexin V staining. Transactivation of the epidermal growth factor (EGF) receptor was investigated by blockade of the receptor in the presence of ET-1, measurement of levels of phosphorylated EGF receptor in the presence of ET-1, and comparing the effects of ET-1 and EGF on cell proliferation.**

Results: **ET-1 significantly stimulated growth of all cell lines via ET_A receptors. ET-1 stimulated DNA replication, not apoptosis. ET-1-stimulated growth was inhibited by antagonism of pertussis toxin-sensitive G proteins, PI3K and PKC. Inhibition of the EGF receptor reduced the effect of ET-1. ET-1 increased levels of phosphorylated EGF receptor via the ET_A receptor.**

Conclusion: **ET-1 increased DNA replication in colorectal cancer cells via the ET_A receptor. This mitogenic action was mediated via pertussis toxin-sensitive G proteins, PI3K, PKC and transactivation of the EGF receptor.**

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Introduction

Colorectal cancer is both the second commonest cancer and the second commonest cause of cancer death in Europe; it was responsible for 203 700 deaths in 2004[1]. Despite improvements in surgical and oncological treatment, survival at 5 years remains only 50 per cent.

Endothelin (ET) 1 is a small vasoactive peptide[2], increased levels of which have been detected in plasma and tissue samples from patients with various solid tumours, including colorectal cancer[3]. Raised plasma levels of big ET-1, a precursor of the active peptide, are an independent indicator of poor prognosis for patients with colorectal cancer[4]. Human colorectal, breast, stomach and prostate cancer cell lines have been shown to produce high levels of ET-1 *in vitro*[5,6]. ET-1 in turn stimulates mitogenesis in colorectal, ovarian and prostate cancer cell lines[7–9].

The effects of ET-1 are mediated by two specific G protein-coupled receptors, ET_A and ET_B[10,11]. Epithelial cancers such as colorectal, renal and ovarian tumours upregulate ET_A and downregulate ET_B receptors[12–14], whereas breast and lung cancers upregulate ET_B and downregulate ET_A receptors[15,16]. The latter are thought to use ET-1 in a paracrine role, stimulating angiogenesis and stromal development rather than stimulating cancer cell growth directly[17]. It has been demonstrated previously that ET-1 stimulates increased net cell growth in colorectal cancer cell lines via the ET_A receptor[7]. A mitogenic growth mechanism has since been challenged and an antiapoptotic

mechanism was suggested for the ET-1 growth response[18]. The first aim of this study was to determine the relative role of apoptosis and mitosis in ET-1-driven cell growth.

A further aim was to identify some of the intracellular pathways involved in this mitogenic action. Cell signalling pathways following activation are complex. The activated G protein can phosphorylate multiple pathways including phosphoinositide 3-kinase (PI3K) cascades that can trigger activation of calcium channels, as demonstrated in both glioma and Chinese hamster ovary cells[19,20]. The effects of ET-1 have also been shown to be mediated partly by phospholipase C and protein kinase C (PKC) in Chinese hamster ovary cells[21], and by PKC in ovarian cancer cell lines[22]. The interaction between ET-1 signalling and the mitogen-activated protein kinase (MAPK) pathway is of great interest. The MAPK pathway is considered to be the major pathway in cancer cell proliferation and has been suggested to interact with ET-1 pathways at a number of levels. The small G protein Ras can be activated by raised intracellular calcium or by PI3K activation[23], the downstream signalling protein Raf-1 can be activated by PKC[24], and tyrosine kinase receptors such as epidermal growth factor (EGF) receptor can be transactivated by ET-1[25]. The complexity of these ET-1 pathways is being clarified as multiple sites of ET_A and ET_B receptor post-translational modification are identified[26].

Materials and methods

Cell culture

Three human colorectal cancer cell lines were used: LIM1215 (derived from a human non-polyposis colonic cancer), HT29 (derived from a primary adenocarcinoma) and SW620 (derived from a metastatic adenocarcinoma). SW620 cells do not express the EGF receptor[27]. Cells were obtained and routinely cultured as described previously[7].

Growth assays

Cells were seeded in 96-well plates (20000/well) and cultured with 10^{-7}–10^{-11} mol/l ET-1/well (Sigma, Poole, UK) as described previously[7]. In combination experiments, cells were cultured with ET-1 (10^{-8} mol/l) and either BQ123 (Sigma), a selective ET_A receptor antagonist, or BQ788 (Alexis Corporation, Nottingham, UK), a selective ET_B receptor antagonist (from 10^{-7} to 10^{-10} mol/l). Cells were also incubated with ET-1 and various concentrations of antagonists: pertussis toxin (Sigma), an antagonist of G protein subtypes Gi and Go, wortmannin (Sigma), an inhibitor of PI3K, tyrphostin AG1478 (Calbiochem, Nottingham, UK), an antagonist of the EGF receptor kinase domain, or GF 109203X (Calbiochem), a PKC inhibitor. HT29 cells were cultured with ET-1 and human recombinant EGF (Sigma) to assess their combined effect on cell growth. Effects on cell growth at 48 h were measured by the methylene blue assay[7].

Immunocytochemistry

SW620 and LIM1215 cells grown in six-well plates were starved of serum for 24 h. ET-1 (10^{-8} mol/l) and/or ET_A receptor antagonist A-127722 (Alexis Corporation) were added and DNA replication determined by uptake of 5-bromo-2′-deoxyuridine (BrdU) (Dako, Ely, UK). Briefly, cells were incubated in BrdU (1 : 1000 in serum-free medium) for 6 h at 37°C, washed in phosphate-buffered saline, fixed in acid–alcohol at 21°C for 30 min, washed and incubated in reconstituted nuclease/anti-BrdU (Dako) for 1 h. Cells were then incubated in peroxidase antimouse IgG2a for 30 min followed by 3, 3′-diaminobenzidine tetrachloride for 5–10 min. Counts were performed by two independent observers on three experimental repeats (three fields/well, minimum 2000 cells/plate).

Flow cytometry

Cells were cultured in serum-free medium with ET-1 and/or specific endothelin receptor antagonists. After disaggregation, cells were washed in Annexin V binding buffer (BD PharMingen, San Diego, California, USA) and resuspended in 1 ml HEPES buffer and 5·5 μl Annexin V–FITC (BD PharMingen). They were incubated in the dark for 20 min at 21°C, and 100 μl of 50 μl/ml propidium iodide (Sigma) was then added. Cells were analysed by flow cytometry, using a FACSCalibur (Bector Dickinson, San José, California, USA); Annexin–FITC fluorescence was measured in the FL1 channel (530/30 bandpass filter) and propidium iodide in FL2 (585/42 bandpass filter). Twenty thousand events were collected. Apoptotic cells were defined as being Annexin positive and propidium iodide negative, whereas late apoptotic/necrotic cells were positive for both markers.

Determination of protein expression

LIM1215 cells were grown to confluence in serum-containing medium in six-well plates. After serum starvation for 12 h, cells were washed and preincubated with antagonists for 1 h. Following 24 h exposure to ET-1 and/or antagonists, cells were lysed with 300 μl mammalian cell lysis/extraction reagent (Sigma) and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis on 12 per cent gels[28]. The separated proteins

were transferred on to nitrocellulose membranes (30V for 90 min). Membranes were blocked by incubation with 5 per cent non-fat milk (in 0·2 per cent Tween-20/PBS) for 1 h and antigens were detected using Tyr^{845} phospho-specific EGF receptor antibody (Calbiochem) diluted 1 : 1000 in PBS. Primary antibody was visualized using an antirabbit secondary IgG–biotin conjugate (Vector Laboratories, Burlingame, CA, USA). Antigen–antibody complexes were detected by incubation with avidin biotinylated enzyme complex (ABC) reagent (Vector Laboratories) using the enhanced chemiluminescence substrate kit (Amersham, Peterborough, UK). Optical densities (means values from four films) were recorded with a densitometer.

Statistical analysis

Analysis of all cell culture experiments was carried out using one-way ANOVA. If statistically significant, *post hoc* analysis was undertaken using either one-way Dunnett's test, set at $P < 0{\cdot}050$, for ET-1 dose–response experiments, or Tukey's honestly significant difference test, set at $P < 0{\cdot}050$, for other analyses. Means of at least six independent repeats for all experiments were analysed, unless stated otherwise. Graphical data are shown as mean(s.d.). Immunocytochemical data are presented as median (range) and were analysed using the Mann–Whitney U test, with $P < 0{\cdot}050$ considered statistically significant.

Results

Effect of endothelin 1 on cell growth

ET-1 had a significant, dose-dependent, positive growth effect on all cell lines (*Table 1*). The effect was maximal at 10^{-8} mol/l, and this dose was used for all subsequent experiments. The mitogenic stimulus tailed off at higher concentrations (10^{-7} mol/l) of ET-1 (data not shown).

Immunocytochemistry

Cells showed significant ET-1-induced increases in DNA proliferation. The median (range) number of SW620 cells that stained for BrdU was 370 (339–386) per 1000 cells counted, increasing to 432 (424–435) per 1000 on addition of ET-1 (16·8 per cent increase; $P = 0{\cdot}040$). For LIM1215 cells, respective figures were 417 (402–443) and 703 (674–715) per 1000 cells (68·6 per cent increase; $P = 0{\cdot}008$). The proportion of positive cells fell to near-control levels in the presence of ET-1 and the ET_A receptor antagonist A-127722: 374 and 364 per 1000 SW620 and LIM1215 cells respectively.

Table 1 Cell growth induced by endothelin 1

	48 h		72 h	
Cell line	Growth increase (%)	*P*	Growth increase (%)	*P*
HT29	23	0·003	19	0·050
SW620	26	0·004	21	0·001
LIM1215	46	< 0·001	33	0·045

Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell number) at 650 nm. Values are percentage increase in growth above control level induced by 10^{-8} mol/L endothelin 1 and represent the mean of six independent experiments.

Flow cytometry

Exposure to ET-1 (10^{-8} mol/l) or a combination of ET-1 and A-127722 did not alter the apoptotic index of the cell lines tested (*Table 2*). The cell distribution profile seen in flow cytograms was consistent with the values obtained, with the four quadrants similarly densely inhabited in control and test groups.

Effects of inhibitors of endothelin 1, G protein and kinases on cell proliferation

The mitogenic effect of ET-1 (10^{-8} mol/l) was significantly inhibited to near-control levels by ET_A receptor antagonism with BQ123 at all doses tested (HT29, $P = 0{\cdot}017$; LIM1215, $P < 0{\cdot}001$; SW620, $P = 0{\cdot}001$). This effect was not seen with the ET_B receptor antagonist BQ788 (data not shown).

Preincubation with the selective G protein antagonist pertussis toxin significantly inhibited the ET-1 growth effect in LIM1215 cells (*Fig. 1*), and similar results were

Table 2 Proportion of apoptotic cells determined by flow cytometry

	Apoptotic cells (ratio of control values)		
Cell line	ET-1	ET-1 + ET_A receptor antagonist	*P*
SW620	0·841	1·148	0·274
LIM1215	0·952	0·994	0·864

Apoptosis levels are expressed as a ratio of control levels in the presence of endothelin (ET) 1 (10^{-8} mol/L) and ET-1 + A-127722 (ET_A receptor antagonist). Data are mean of ten independent repeats.

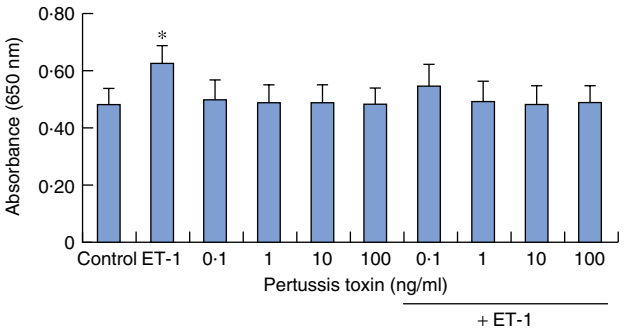

Fig. 1 Effect of G protein inhibition with pertussis toxin on endothelin (ET) 1-stimulated growth of LIM1215 cells. Cells were pretreated for 6 h with pertussis toxin, then with increasing doses of pertussis toxin (0·1–100 ng/ml) with or without ET-1 (10^{-8} mol/l) for 48 h. Cell growth was measured as absorbance at 650 nm using the methylene blue assay. Values are mean(s.d.). Pertussis toxin significantly inhibited ET-1-stimulated cell growth ($P = 0·003$, one-way ANOVA). *$P < 0·050$ (*post hoc* analysis with Tukey's honesty significant difference test) significant *versus* all other groups

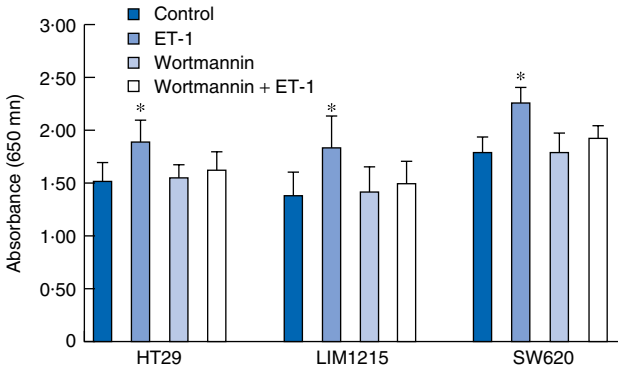

Fig. 2 Effect of the phosphoinositide 3-kinase inhibitor wortmannin on endothelin (ET) 1-stimulated cell growth. Cells were cultured for 48 h with ET-1 (10^{-8} mol/l), wortmannin (5 μmol/l) or both. Values are mean(s.d.). Wortmannin significantly but not completely inhibited ET-1-stimulated growth in HT29 ($P = 0·008$), LIM1215 ($P = 0·040$) and SW620 ($P = 0·008$) cells (one-way ANOVA). *$P < 0·050$ significant *versus* all other groups (*post hoc* analysis with Tukey's honestly significant difference test)

obtained with HT29 ($P < 0·001$) and SW620 ($P = 0·002$) cell lines.

Cells exposed to ET-1 and the PI3K inhibitor wortmannin demonstrated a partial, but significant reduction in the growth effect of ET-1 (*Fig. 2*). The PKC inhibitor GF 109203X significantly inhibited the effect of ET-1 in LIM1215 cells (*Fig. 3*), and similar results were obtained for HT29 ($P < 0·010$) and SW620 ($P = 0·008$) cell lines.

Epidermal growth factor receptor and endothelin 1-stimulated cell growth

Antagonism of EGF receptor kinase activity with tyrphostin AG1478 resulted in significant inhibition of the ET-1 mitogenic effect to near-control levels in HT29 and LIM1215 cells (*Table 3*). In SW620 cells, which do not express the EGF receptor[27], tyrphostin AG1478 had no inhibitory effect on ET-1-stimulated growth.

In HT29 cells ET-1 (10^{-8} mol/l) is equipotent in mitogenic effect to 5 ng/ml EGF. When HT29 cells were stimulated with combinations of both growth factors, an additive/synergistic effect was observed (*Fig. 4*).

Western blots of proteins from LIM1215 cells showed that ET-1 increased the level of phosphorylated EGF

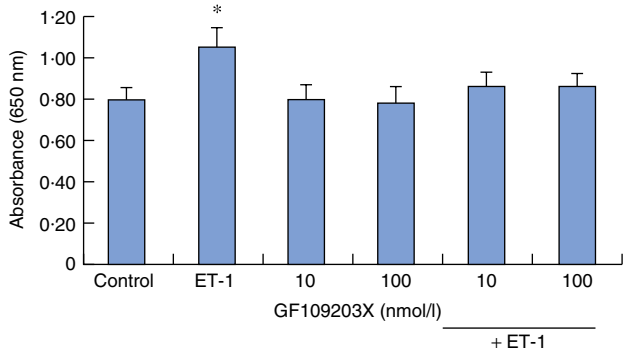

Fig. 3 Effect of protein kinase C antagonism on endothelin (ET) 1-stimulated growth of LIM1215 cells. Cells were cultured for 48 h in the presence of ET-1 (10^{-8} mol/l), GF 109203X or both, at the indicated doses. Values are mean(s.d.). GF 109203X significantly inhibited ET-1-stimulated cell growth ($P = 0·020$, one-way ANOVA). *$P < 0·050$ significant *versus* all other groups (*post hoc* analysis with Tukey's honestly significant difference test)

Table 3 Effect of antagonism of epidermal growth factor receptor on endothelin 1-stimulated cell growth

	Mean absorbance at 650 nm				
	Control	ET-1	Tyrphostin AG1478	ET-1 + tyrphostin AG1478	*P*
HT29	2·08(0·21)	2·55(0·10)	2·13(0·14)	2·11(0·16)	0·048
LIM1215	0·70(0·07)	0·92(0·06)	0·71(0·03)	0·71(0·03)	<0·010
SW620	2·26(0·13)	2·71(0·11)	2·31(0·13)	2·67(0·14)	0·317

Values are mean(s.d.). ET-1 group significantly different from all other groups for HT29 and LIM1215 (one-way ANOVA, $P < 0·050$ at *post hoc* analysis with Tukey's honestly significant difference test). ET-1, endothelin 1.

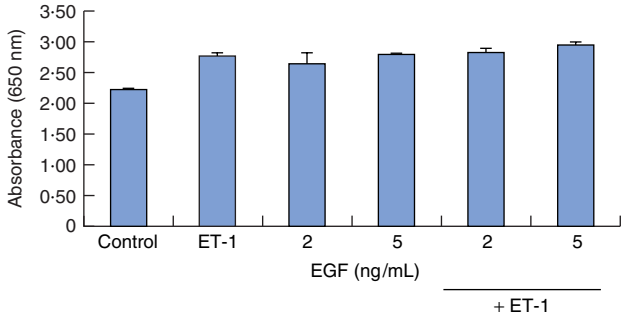

Fig. 4 Effect of epidermal growth factor (EGF) and endothelin (ET) 1 on growth of HT29 cells. Cells were cultured for 48 h in the presence of ET-1 (10^{-8} mol/l), EGF or both, at the indicated doses. Values are mean(s.d.) of eight independent experiments. $P = 0{\cdot}007$ (one-way ANOVA). $P < 0.010$ all groups *versus* control (*post hoc* analysis with Tukey's honestly significant difference test)

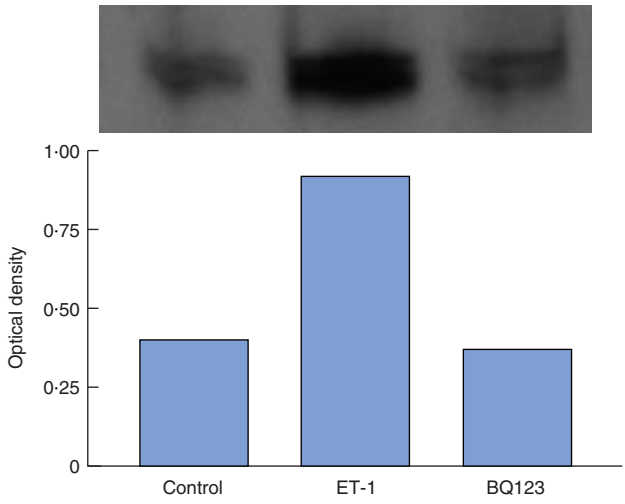

Fig. 5 Effect of endothelin (ET) 1 with or without the ET_A receptor antagonist BQ123 on levels of phosphorylated epidermal growth factor (EGF) receptor in LIM1215 cells. A western blot of phosphorylated EGF receptor protein is shown and the graph beneath shows the mean optical densities of four such blots

receptor, and that this phosphorylation was reduced to control levels by addition of the ET_A inhibitor BQ123 (*Fig. 5*).

Discussion

This study has demonstrated that ET-1, a peptide overexpressed by both adenomas and colonic cancers, stimulates growth of colonic cancer cells[3,29]. This supports findings in other epithelial cancers[7–9]. The tailing off of this growth effect at a high concentration (10^{-7} mol/l) of ET-1 suggests a toxic effect at such levels. These levels appear to be 10 000 times higher than circulating plasma concentrations in patients with colorectal cancer[30]. The positive growth effect is mediated by the ET_A receptor, as shown by the inhibition caused by BQ123 but not BQ788. This is consistent with the finding of upregulation of ET_A and downregulation of ET_B receptors in epithelial tumour tissue[12,17], and mediation of the mitogenic signal by ET_A receptors in epithelial cancer cells[9,13,31].

Changes in cancer cell numbers are due to altered rates of both mitosis and apoptosis. In colorectal cancer cells, the findings of increased BrdU uptake and no change in the level of apoptosis suggest that the increases in cell numbers are the result of a mitogenic effect of ET-1. This has also been demonstrated with thymidine incorporation assays in human ovarian cancer and meningioma cells[8,32], and by flow cytometry in prostate cancer cells[9].

Preincubation of cells with the specific G protein antagonist pertussis toxin completely inhibited the mitogenic effect of ET-1 at doses of 1–100 ng/ml, suggesting that the mitogenic effect of ET-1 in colorectal cancer cells is mediated by pertussis toxin-sensitive G protein subunits Go or Gi. This contrasts with the report of pertussis toxin-insensitive ET-1-stimulated DNA replication in ovarian cancer cells[22]. Work in vascular smooth muscle cells showed that the mitogenic effect of ET-1 mediated via transactivation of the EGF receptor requires a pertussis toxin-insensitive G protein[33]. However, stimulation of the ET_A receptor with ET-1 has been shown to result in receptor coupling with pertussis toxin-sensitive Go α and Gi_3 α subunits[34].

The ET-1 mitogenic stimulus was significantly inhibited by wortmannin in all cell lines, suggesting that PI3K activation provides an additional mechanism whereby ET-1 can mediate proliferation. Incubation of colorectal cancer cells with the PKC antagonist GF 109203X resulted in partial inhibition of the ET-1 growth effect. Interestingly, the inhibitory effect was greater in SW620 cells (lacking EGF receptor), suggesting a greater role for PKC activation in propagation of the ET-1 mitogenic signal in these cells.

Specific EGF receptor kinase antagonism almost totally inhibited the stimulatory effect of ET-1 on cell growth in the two EGF receptor-positive cell lines investigated. There was a marked increase in the level of phosphorylated EGF receptor in the presence of ET-1, which was ameliorated by ET_A receptor blockade. These data suggest a role for EGF receptor transactivation in the propagation of the ET-1 mitogenic signal, as previously demonstrated in human ovarian cancer cells[25]. The results also suggest that the combined mitogenic effect of ET-1 and EGF is

additive, rather than synergistic, in keeping with findings in both prostate[9] and ovarian[22] cancer cells. This effect may be determined by the finite bioavailability of downstream effectors activated by these growth factors, or interactions between specific components of the signalling pathways.

This study has confirmed the role of ET-1 as a mitogen in colorectal cancer cells and identified some of the underlying intracellular mechanisms, highlighting the potential for interaction with the MAPK pathway.

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35 years ago

Nasal carriage of *Staphylococcus aureus* in a general surgical unit

Still a 'hot topic' and no less important nowadays.

Nasal carriage of staphylococci was found in 50 per cent of in-patients in a general surgical unit and in 26 per cent of out-patients. While 32 per cent of in-patients carried resistant organisms in their noses a high percentage of out-patients (17 per cent) also were carriers of resistant organisms. Eighty per cent of staff carried staphylococci in their noses, 67 per cent being resistant organisms. Although the 'hospital staphylococcus' was found in 5 per cent of patients and 15 per cent of attending staff, only I per cent of in-patients and none of the out-patients or staff carried multiple-resistant organisms. Age does appear to have some bearing on the carriage of bacteria in the anterior nares. In contrast to other studies, this work failed to reveal any relationship between wound infection and nasal carriage of staphylococci. These findings suggest that general dissemination of bacteria in the hospital environment may be of greater importance than autoinfection as a cause of wound sepsis.

Doig CM. Nasal carriage of *Staphylococcus aureus* in a general surgical unit. *Br J Surg* 1971; **44**: 55–68
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