The differential effects of statins on the metastatic behaviour of prostate cancer

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**BACKGROUND:** Although statins do not affect the incidence of prostate cancer (CaP), usage reduces the risk of clinical progression and mortality. Although statins are known to downregulate the mevalonate pathway, the mechanism by which statins reduce CaP progression is unknown.

**METHODS:** Bone marrow stroma (BMS) was isolated with ethical approval from consenting patients undergoing surgery for non-malignant disease. PC-3 binding, invasion and colony formation within BMS was assessed by standardised *in vitro* co-culture assays in the presence of different statins.

**RESULTS:** Statins act directly on PC-3 cells with atorvastatin, mevastatin, simvastatin (1 μM) and rosuvastatin (5 μM), but not pravastatin, significantly reducing invasion towards BMS by an average of 66.68% (range 53.93–77.04%; *P* < 0.05) and significantly reducing both number (76.2 ± 8.29 vs 122.9 ± 2.48; *P* = 0.0055) and size (0.2 ± 0.0058 mm² vs 0.27 ± 0.012 mm²; *P* = 0.0019) of colonies formed within BMS. Statin-treated colonies displayed a more compact morphology containing cells of a more epithelial phenotype, indicative of a reduction in the migrational ability of PC-3 cells. Normal PC-3 phenotype and invasive ability was recovered by the addition of geranylgeranyl pyrophosphate (GGPP).

**CONCLUSION:** Lipophilic statins reduce the migration and colony formation of PC-3 cells in human BMS by inhibiting GGPP production, reducing the formation and the spread of metastatic prostate colonies.

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Prostate cancer (CaP) is the second most common malignancy in men worldwide, with 910 000 new cases diagnosed in 2008 (Ferlay et al, 2010). CaP has a predilection to metastasise to the bone marrow stroma (BMS), and development of CaP bone metastases almost invariably result in CaP-related mortality (George, 1988). The metastatic process is a complex, multistep process, which can be modelled with modified *in vitro* invasion chambers utilising human primary BMS (Lang et al, 1998; Scott et al, 2001; Hart et al, 2005). These *in vitro* models not only allow the determination of the mechanism of CaP metastasis to the BMS but enable the elucidation of how therapeutic agents may interfere with the metastatic process.

Recent evidence links lipid metabolism and statin use with the behaviour of CaP. Large scale epidemiological data showing lower rates of CaP progression in patients taking statins (Platz et al, 2006) have been consolidated by reports showing that CaP incidence in screened (Murtola et al, 2010) and non-screened (Breau et al, 2010) populations is reduced in men taking these drugs and that individuals with lower cholesterol levels had lower rates of high risk disease (Platz et al, 2008). Further clinical data from histological analysis of large case after prostatectomy series showed less aggressive features in men taking statins (Loeb et al, 2010) and in patients undergoing radiotherapy for CaP indices of CaP treatment failure were reduced in men taking statins, especially in those with high risk features (Gutt et al, 2010).

The basis for these observations is poorly understood but the effects probably relate to the pleiotropic actions of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) inhibitors on cellular behaviour, cell-cell interaction and cellular motility in relation to lipid metabolism (Stancu and Sima, 2001). To better understand this process, we have studied the effects of different statins in our in-house, well-categorised models of CaP behaviour, evaluating the differential effects of specific statins on cellular binding, migration and early cellular survival in human BMS.

**MATERIALS AND METHODS**

**Cell culture**

PC-3 (ATCC-LGC, Teddington, UK) cells were cultured in HAM’S F12 media supplemented with 7% foetal calf serum (FCS) and 2 mM L-glutamine at 37 °C, 5% CO₂ in air. PC3-GFP were cultured as for PC-3 except for addition of 0.15 mg ml⁻¹ hygromycin B. Human BMS was obtained from volunteers undergoing surgery for benign disease and cultured according to Coutinho et al (1993). Briefly, 2 × 10⁶ cells ml⁻¹ in long-term bone marrow culture medium

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100 mM before storing at 4°C. The pH was brought to 7.0 by HCl and diluted to a final concentration of 100 mM. Mevalonate was dissolved in chloroform at 100 mM. Mevalonate, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were dissolved in methanol at a final concentration of 1 mg ml⁻¹. Cholesterol was dissolved in chloroform at 100 mM. Mevalonate was made up in water at a stock concentration of 10 mg ml⁻¹/C₅O.

Cholesterol was dissolved in dimethyl sulphoxide (DMSO) and pravastatin (Sigma-Aldrich, Poole, UK), mevastatin, and simvastatin (Sigma-Aldrich, Sigma-Aldrich, Poole, UK) were dissolved in dimethyl sulfoxide at a stock concentration of 10 mg ml⁻¹/C₅O. New concentrations of statins, including 5 μM rosvastatin, 1 μM pravastatin, 1 μM simvastatin, and 5 μM rosuvastatin were made according to the manufacturer’s instructions. The effect of statins on PEC within the lipid-rich environment of the BMS is unknown. We therefore compared the effect of statins on PEC within the lipid-rich environment of the BMS.

Effect of simvastatin on BMS invasion

Simvastatin treatment of BMS reduced invasion as compared with controls (84 ± 8.8%; P = 0.18806 vs 80 ± 10.57%; P = 0.14755), this did not reach significant levels (Figure 1A). All statins, except pravastatin, induced a similar and marked reduction in PEC binding to BMS (9.37% ± 0.21212). Activation of simvastatin according to the manufacturer’s instructions had no effect on the ability of BMS to induce PEC invasion; although atorvastatin and simvastatin treatment of BMS reduced invasion as compared with controls (9.37% vs 100%, P = 0.0201 and 25.6% vs 100%, P = 0.0496 for rosuvastatin and simvastatin vs BMS + vehicle control, respectively), with 5 μM rosuvastatin reducing invasion to levels similar to those of BMS invasion by lipophilic statins

RESULTS

Reduction of BMS invasion by lipophilic statins

We have previously shown that BMS is the most potent chemoattractant for metastatic PECs and that the metastatic process can be modelled using primary human BMS co-cultures (Hart et al., 2005; Brown et al., 2006). We therefore addressed three different questions: do statins affect the BMS microenvironment (Figure 1A), the PECs directly (Figure 1B) or the interaction between BMS and PECs (Figure 1C). Statins did not affect the ability of BMS to induce PEC invasion; although atorvastatin and simvastatin treatment of BMS reduced invasion as compared with controls (84 ± 8.8%; P = 0.18806 vs 80 ± 10.57%; P = 0.14755), this did not reach significant levels (Figure 1A). All statins, except pravastatin, induced a similar and marked reduction in PEC invasion towards BMS (P > 0.05), averaging 66.68% of control (range 54–77%; P < 0.05). Bone marrow stroma pretreatment (Figure 1C) did not provide additional benefit to treating PECs alone (58.86%; P > 0.05 of PC-3’s invading compared with controls), suggesting a direct effect on the PC-3 cells and not on the BMS ‘soil’.

The effect of statins was more significant in the presence of a confluent BMEC/Matrigel barrier, which models the blood microenvironment. Both 5 μM rosuvastatin and 1 μM simvastatin (Figure 1D) completely inhibited direct invasion towards BMS (9.37% vs 100%, P = 0.0201 and 25.6% vs 100%, P = 0.0496 for rosuvastatin and simvastatin vs BMS + vehicle control, respectively), with 5 μM rosuvastatin reducing invasion to levels similar to those of BMS invasion by lipophilic statins

Supplementary Figure 1).

Effect of statins on PEC binding to BMS

When malignant PECs enter the BMS, they migrate towards and bind to niches within the BMS before proliferating and forming viable colonies (Brown et al., 2010). We sought to assess the inhibitory effect of statins on this process. Incubation of PECs with statins for up to 120 min did not alter the ability of PECs to bind to BMS (Figure 2).

Reduction of PEC colony formation in BMS by lipophilic statins

Previous studies have documented the antiproliferative effect of high-dose statin treatment of PECs. However, the antiproliferative effect of statins on PEC within the lipid-rich environment of the BMS is unknown. We therefore compared the effect of statins on...
PEC colony formation and proliferation in isolation with clonogenic assay or in BMS co-culture.

Pravastatin, at doses up to 100 μM, had no effect on PC-3 colony formation or cellular proliferation in either clonogenic or BMS co-culture assays (Figure 3A). However, the lipophilic statins and rosuvastatin all had a significant effect on colony formation in both assays and cellular proliferation. Lipophilic statins and rosuvastatin significantly reduced the number of colonies to a similar degree in clonogenic assays and BMS co-culture (Table 1), with simvastatin being the most potent (reduction of 75.44% and 49.44%, respectively). Bone marrow stroma colonies were smaller than controls (0.19 ± 0.025 mm², 0.21 ± 0.03 mm², 0.21 ± 0.03 mm², 0.19 ± 0.03 mm²; P < 0.05) resulting in a reduction of the total PEC area (12.8 ± 3.34 mm², 20.7 ± 4.5 mm², 18.68 ± 4.1 mm², 14 ± 3.8 mm²) in co-cultures treated with rosuvastatin, atorvastatin, mevastatin and simvastatin, respectively (Figure 3B). There was no difference (P > 0.05) in the colony size, number or overall PEC load between the four statins.

Typically, PC-3 colonies in BMS co-culture formed large diffuse colonies of migrating PECs within the BMS. Cells displayed a
mesenchymal morphology, being teardrop shaped, with ruffled leading edges of lamellipodia in the absence of therapeutic agents or in the presence of DMSO vehicle control (Figure 3C, control). The lipophilic statins induced significant morphological changes in the BMS colonies. Prostate epithelial cell colonies were more tightly packed, containing fewer cells than controls. At a higher magnification they displayed differing morphology compared with the mesenchymal morphology seen in control co-cultures, with
Mechanism of inhibition of metastatic behaviour by statins

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Mechanism of statin inhibition of invasion

Statins act by blocking the conversion of HMG-CoA to mevalonic acid through inhibition of HMG-CoA reductase (Figure 4A). We therefore sort to recover the invasion by supplying the downstream metabolites mevalonate, cholesterol or the prenylating agents GGPP and FPP. Treating PC-3-GFP cells with either GGPP or mevalonate had no significant effect on their ability to invade towards BMS ($P = 0.259$ and 0.619, respectively). Both cholesterol and FPP reduced PC3-GFP invasion towards BMS, but this reduction was not significant (75.51% ± 2.25%; $P = 0.158$ and 54.9% ± 8.24%; $P = 0.056$, respectively). PC3-GFP cells were treated with mevalonate, GGPP, FPP or cholesterol before treatment with PECs being more rounded, without obvious lamellipodia at the cell’s leading edge. Although some cells retained their teardrop appearance, the leading edge did not possess lamellipodia and the tails were distorted, appearing longer, wider and curved (Figure 3C). These morphological effects were not seen with pravastatin.

Table 1  Effect of stains on prostate epithelial colony formation in BMS co-culture

| Mean colony number | s.e. | Fold reduction | P-value |
|-------------------|------|---------------|---------|
| No drug control   | 118  | 12.84         |         |
| DMSO low control  | 150.7| 19.97         |         |
| DMSO high control | 125  | 13.54         |         |
| Rosuvastatin      | 61.2 | 13.76         | 2.04    | 0.0095  |
| Atorvastatin      | 94.6 | 13.91         | 1.59    | 0.055   |
| Mevastatin        | 85.8 | 13.56         | 1.76    | 0.0315  |
| Simvastatin       | 63.2 | 4.72          | 2.38    | 0.0117  |
| Pravastatin       | 125  | 27.79         | 0.94    | 0.7928  |

Abbreviations: BMS = Bone marrow stroma; DMSO = dimethyl sulphoxide; PC-3 colony formation after statin treatment in BMS co-culture assay. PC-3 cells were treated with 1 μM atorvastatin, mevastatin, simvastatin, 5 μM rosuvastatin or 10 μM pravastatin daily for 8 days in BMS co-culture. Colonies >32 cells were counted. Table 1 shows the mean number of colonies, the fold reduction in numbers as compared with the relevant vehicle controls and the significant difference between treatments and their respective vehicle controls.

Figure 4  Geranylgeranyl pyrophosphate recovers statin-inhibited invasion towards BMS. (A) Diagrammatic representation of the mevalonate pathway highlighting the metabolic blocks of statins and bisphosphonates. (B) Histogram depicting the effect of the addition of the mevalonate pathway metabolites (mevalonate, GGPP, FPP or cholesterol) downstream of the statin block in a BMS co-culture assay utilising a BMEC barrier layer. (Bi) Invasion recovery assay in the presence of 1 μM ROS. (Bii) Invasion recovery assay in the presence of 1 μM SIM.
either 1 μM simvastatin or 5 μM rosuvastatin, and invasion through a BMEC/Matrix barrier towards untreated BMS was assessed. The addition of mevalonate to the invasion assay completely restored/enhanced the invasion towards BMS (66.67% ± 13.26%; P = 0.1126 and 117.42% ± 17.79%; P = 0.0033 for rosuvastatin and simvastatin, respectively). However, of the addition of downstream metabolites, only GGPP was able to completely restore rosuvastatin-treated invasion towards BMS (82.42 ± 5.52%; P = 0.229). Addition of GGPP to simvastatin-treated cells resulted in an enhanced invasion as compared with the statin-free controls (122 ± 7.83%; P = 0.0185). Addition of either FPP or cholesterol did not affect the ability of rosuvastatin or simvastatin to inhibit invasion towards BMS. Activation of simvastatin had no effect on the invasion profile, with both GGPP (111.5 ± 8.59%; P = 0.9079) and mevalonate (67.04 ± 8.03%; P = 0.0547) restoring the invasive ability of PC3-GFP cells towards BMS (P = 0.1391 and P = 0.2267 comparing activated to not activated simvastatin in the presence of GGPP or mevalonate, respectively) (Supplementary Figure 2).

**DISCUSSION**

This is the first study to report the comparative effects of different statins on CaP cellular migration towards and within human BMS. The results demonstrate a clear effect on CaP migration towards and through BMS and on malignant PEC’s ability to grow clonally in that location. However, this effect was limited to the lipophilic statins and was not seen with the hydrophilic statin, pravastatin. The differential effect of the two basic subtypes of this class of drug has been well described; lipophilic statins diffuse across cellular membranes and exert their metabolic effects in the liver and other tissues; hydrophilic statins require active transport across the cell membrane in order to exert their actions intracellularly. This action of hydrophilic agents is therefore predominantly hepatic and not peripheral (Stancu and Sima, 2001; Garwood, 2010) and this fact has significant consequences for the effects observed in neoplasms such as breast and other cancers (Campbell et al, 2006; Kotamraju et al, 2007; Koyuturk et al, 2007). This differential action was clearly evident in the results presented herein and may be an important consideration when interpreting data from population studies of the effect of statins or when planning statin-based prevention trials in CaP.

Epidemiological studies have shown that although statin use does not affect CaP incidence, it does reduce the risk of clinical progression and CaP-related mortality; the mechanisms of this are unknown. Statins can induce apoptosis in a range of malignant neoplasms such as breast and other cancers (Campbell et al, 2007). This differential action was clearly evident in the results presented herein and may be an important consideration when interpreting data from population studies of the effect of statins or when planning statin-based prevention trials in CaP.

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HMG-CoA reductase inhibition. This affects membrane integrity, cell signalling, protein synthesis and cell cycle progression. Disruption of these processes by statins may result in inhibition of tumour initiation, growth and metastasis (Boudreau et al, 2007). Mevalonate is the precursor of the isoprenoids FPP and GGPP, essential for the translocation of signalling proteins, such as the G-proteins Ras and Rho to the plasma membrane, where they mediate signal transduction by controlling cell differentiation, proliferation and migration (Hager et al, 2006). Addition of GGPP, unlike cholesterol or FPP, to our invasion assays completely restores the ability of PC-3 cells to invade towards the BMS, suggesting that the lipophilic statins act by preventing the translocation of nascent signalling proteins, which require geranylgeranylation, to the cell surface. This would lead to a reduction in signalling proteins at the cell surface and thereby preventing the cell from detecting the invasive stimulus from the BMS or BMS adipocytes (Brown et al, 2010).

Further studies support the notion that the mevalonate/G-protein axis is important. Zhao et al (2010) demonstrated that 10 μM lovastatin inhibited EGFR dimerisation and signalling via AKT and its downstream targets, 4E-binding protein 1 and S6 kinase 1, in a GGPP-dependent manner. Lovastatin also induced cytoskeletal disorganisation associated with increased inactive RhoA expression, which lacked the GGPP tag. Co-incubation with mevalonate or GGPP restored RhoA activity and EGF signalling. This suggests that inhibition of invasion towards and through BMS is more likely to arise from isoprenoid loss rather than loss of endogenous cholesterol production by the CaP cells. This latter factor may be critical; the ability of the cancer cell to move is fundamental to metastasis and its inhibition prohibits metastatic CaP survival in BMS (Lang et al, 1998). In vitro studies have demonstrated the critical importance of the Rho/Rac axis in mesenchymal to amoeboid transition and invasion/migration (Sanz-Moreno et al, 2008); these steps are fundamental to progression in prostate and other cancers. Thus, important elements of metastatic behaviour are potentially inhibited by lipophilic statins and these anti-migrational effects alone may be responsible for the CaP-related observations presented herein and reported clinically (Platz et al, 2006; Gutt et al, 2010).

Here we have used validated models of malignant prostate epithelial invasion to examine the effects of statins on the migratory pathway in CaP metastasis. We have shown for the first time in this laboratory setting that statins act directly on malignant PEC and block the formation of GGPP from HMG-CoA. Loss of GGPP leads to a significant reduction in the ability of malignant PEC to invade towards and through BMS and so reduce their ability to form colonies within the BMS. This statin effect is not universal across all classes of statins as the hydrophilic statin pravastatin had no effect on the PC-3 cell line in our models. In summary, statins appear to act on two key components on CaP metastasis; reduce tumour growth, possibly through the reduction of cholesterol but also, as described here, through inhibiting geranylgeranyl prenylated pathways required for transendothelial migration and BMS invasion.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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