Osteopontin expression correlates with adhesive and metastatic potential in metastasis-inducing DNA-transfected rat mammary cell lines

A metastatic phenotype can be induced in benign rat mammary cells (Rama 37 cells) by transfecting them with metastasis-inducing DNAs (Met-DNAs). Stable transfection of Met-DNAs increases the level of the metastasis-associated protein, osteopontin. Randomly picked clonal cell lines have been established from the pool of Rama 37 cells transfected with one metastasis-inducing DNA, C9-Met-DNA. In these cell lines, moderate correlation is observed between the copy number of C9-Met-DNA and their metastatic potential (linear regression coefficient, $R^2 = 0.48$). A very close correlation is observed between the cell lines’ metastatic potential in vivo and the osteopontin mRNA levels in vitro ($R^2 = 0.74$), but not with another metastasis-associated protein in this system, S100A4 ($R^2 = 0.21$). A close correlation is also observed between osteopontin mRNA levels and the adhesive potential ($R^2 = 0.91$) of the cells, but not with their growth rate in vitro ($R^2 = 0.03$). These observations support the previous suggestion that osteopontin is the direct effector of C9-Met-DNA and that the presence of C9-Met-DNA is necessary, if not sufficient, for the induction of metastasis in vivo in this system. Additionally, these results suggest that Rama 37 cells with increased osteopontin mRNA levels become metastatic not through an increased growth rate, but through an increase in cellular adhesiveness.

Keywords: osteopontin mRNA; transfected cells; adhesion; rat mammary metastasis
In this report, nine clonal cell lines have been established from the original pool of C9-Met-DNA-transfected Rama 37 cells and the molecular and cellular characteristics of each of these clonal cell lines have been investigated. The results now show that osteopontin is the direct effector of metastasis in this system. Additionally, a direct correlation between osteopontin mRNA levels, metastatic potential and cellular adhesion has been shown for the first time, leading to the suggestion that osteopontin induces metastasis in Rama 37 cells, at least in part, by a process reflected in their increased adhesiveness on plastic substrata and not by providing any growth advantage over the untransfected Rama 37 cells.

MATERIALS AND METHODS

Cell culture

All cell lines were maintained as described previously (Dunnington et al., 1983) in a humidified atmosphere of 90% (v v\(^{-1}\)) air and 10% (v v\(^{-1}\)) CO\(_2\) in routine medium (RM) (Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Paisley, Scotland) with 5% (v v\(^{-1}\)) foetal calf serum (FCS) (Sigma, Dorset, UK), 50 ng ml\(^{-1}\) insulin and hydrocortisone). The control cell line Rama 800 was obtained from a transplantable rat mammary tumour (Jamieson and Rudland, 1990). Passaging of cells at a 1:8 dilution was carried out at 70–80% confluence using trypsin in EDTA, 0.05% (v v\(^{-1}\)).

Establishment and testing of clonal cell lines

Clonal cell lines were established from the pool of Rama 37 cells transfected with C9-Met-DNA by plating approximately 10 000 cells in 9 cm diameter dishes. After 1 week, colonies derived from single cells were harvested by placing over the colony a 4 mm diameter disc of Whatman filter paper soaked in trypsin 0.05% (v v\(^{-1}\)) in EDTA and incubating for 10 min at 37°C. The discs with the cells attached were subsequently placed in one well of a 24-well plate in cloning medium (CM) (45% (v v\(^{-1}\)) DMEM (Gibco BRL), 10% (v v\(^{-1}\)) FCS (Sigma), 45% (v v\(^{-1}\)) RPMI (Gibco BRL), 50 ng ml\(^{-1}\) hydrocortisone and insulin). When there were sufficient cells to be plated into 4, 9 cm diameter Petri dishes, they were transferred and maintained in RM. Plating efficiency was determined by plating the cell lines at a known density (20 000–30 000 cells per dish) in conditioned media generated from the RM of cells that had been growing exponentially in a 9 cm diameter plate for 3 days. After plating and incubation for 15 min at 37°C to allow cells to adhere to the plate, the cells were removed from the plate with trypsin/EDTA solution and counted in a Coulter counter. Plating efficiency was calculated as the percentage of cells that had adhered after 15 min. Three independent experiments were carried out in triplicate and error bars represent the standard deviation of the three separate experiments. Cell growth assays were carried out by plating a known density of cells (5–10 000 per well) in 24-well plates. Every day for 6 days, cells in a set of triplicate wells were completely removed by trypsinisation and counted in a Coulter counter. Growth curves were plotted and the gradients of the curves were obtained using linear regression analysis. Growth rates were subsequently represented as a percentage of that for the Rama 37 cell line. Experiments were carried out three times in triplicate and the error bars represent the standard deviation of three separate experiments.

Assay for metastasis

Metastasis assays were carried out as described previously (Dunnington et al., 1983). Cells (2 × 10\(^6\)) to be assayed, suspended in 0.2 ml phosphate-buffered saline (PBS), were injected into the right inguinal mammary fat pads of approximately 20 female Furth Wistar rats of 6–10 weeks of age. Animals were observed every 3 days and killed after 3 months. The primary tumour and lungs of each animal were then fixed in Methacarn (60% (v v\(^{-1}\)) methanol, 10% (v v\(^{-1}\)) acetic acid and 30% (v v\(^{-1}\)) Inhibisol) and embedded in paraffin wax. Sections of 5 µm were then cut and stained with haematoxylin and eosin. Analysis of the stained lung sections allowed individual animals to be scored positive or negative for lung metastasis by two observers. Metastatic potential of the cell lines was expressed as the percentage of tumour-bearing animals exhibiting lung metastases. Animals were maintained under UK Home Office Project Licence no. 40/1515 to Professor PS Rudland in accordance with the guidelines set down by the United Kingdom Coordinating Committee for Research Cancer.

Northern and Southern blotting

DNA and RNA from the cell lines were isolated by ultracentrifugation through caesium chloride gradients of guanine isothiocyanate cell extracts, as described previously (Coombs et al., 1990; Anandappa et al., 1994). Southern transfer was carried out as described in Sambrook et al. (1989). Ten µg of EcoRI-digested genomic DNA was resolved on a 0.8% (w v\(^{-1}\)) agarose gel and blotted overnight on to Hybond-N membrane (Amersham Pharmacia, Buckinghamshire, UK) in 20 × SSC. DNA was fixed on the membrane by exposure to UV light. RNA for Northern blotting was resolved on a 0.8% (w v\(^{-1}\)) formaldehyde agarose gel and blotted onto Hybond-N membrane (Amersham Pharmacia) in 10 × SSC. RNA was fixed on the membrane by exposure to UV light. Probes radioactively labelled with \(\alpha\)-\(\text{P}\)dCTP (ICN, Hampshire, UK) (specific activity >1 × 10\(^{6}\) d.p.m. µg\(^{-1}\) DNA) were generated for Northern and Southern blotting using a random primed labelling kit (Roche Molecular Chemicals, Hertfordshire, UK). The 1500bp osteopontin cDNA probe was obtained by digestion of the pBKCMV-OPN plasmid (Oates et al., 1996) with restriction enzymes XbaI and KpnI. The 300 bp S100A4 cDNA was a PCR product of full-length rat S100A4 cDNA obtained from Dr I Anandappa (University of Liverpool, UK). The 1000 bp C9-Met-DNA probe was obtained by digestion of the pKS-C9-Met-DNA plasmid (Chen et al., 1997) with HindIII. Hybridisation for Northern and Southern blots was carried out at 42°C in formamide buffer (5 × SSC, 50% (v v\(^{-1}\)) formamide, 1 × Denhardt’s solution (Sambrook et al., 1989). 5% (w v\(^{-1}\)) dextran sulphate and 3 µg ml\(^{-1}\) sonicated salmon sperm DNA). After washing the membranes consecutively with: (1) 2 × SSC, 0.1% (w v\(^{-1}\)) sodium dodecyl sulphate (SDS); (2) 0.2 × SSC, 0.1% (w v\(^{-1}\)) SDS; and (3) 0.1 × SSC, 0.1% (w v\(^{-1}\)) SDS, hybridisation signals were visualised by exposing the wrapped membrane to autoradiographic film for 6–72 h with an intensifying screen. Hybridisation signals were quantified using a Molecular XL 77CE CCD video camera and MacIntosh IMAGE and PHOTO computer packages (NIH, Bethesda, MD, USA). C9-Met-DNA copy number was determined by comparing the intensity of C9-Met-DNA hybridised bands from the Rama cells to plasmid copy number controls on the same membrane. Although the amount of plasmid DNA and cellular DNA loaded onto the gel was different, comparison of relative intensities of hybridised bands of plasmid DNA and cellular DNA give a reliable estimate of copy number (Sambrook et al., 1989), as described previously (Davies et al., 1994; Oates et al., 1996; Chen et al., 1997). Equal loading of cellular DNA of the transformant and parental cell lines was indicated by equal sample intensity on the gel following electrophoresis and staining with ethidium bromide. There were no higher molecular weight bands hybridising to the ODN cDNA probe, indicating that digestion of genomic DNA by EcoR1 was complete in all cases. Osteopontin and S100A4 mRNA levels were normalised for differences in loading/transfer by dividing the intensity of the cDNA hybridised band by the intensity of the ethidium bromide-stained 28S ribosomal RNA band, rather than the intensity of an actin cDNA-hybridised band.
which could have possibly had its expression changed by C9-Met-DNA.

Statistical analysis
The Student’s t-test and linear regression analysis to determine correlation coefficients were carried out using the Microsoft EXCEL package. The significance of the latter fit to a straight line was calculated, $P$ values of less than 0.05 were considered statistically significant.

RESULTS

Metastatic potential of clonal cell lines

Nine clonal cell lines were established from the original pool of Rama 37 cells transfected with C9-Met-DNA, they were termed R37C9VM followed by clone numbers. Within the set of clonal lines, various cellular morphologies were observed, ranging from tightly clustered cuboidal to a more elongated morphology (Table 1). Eight of the nine clonal cell lines produced primary tumours in all animals. The exception was the cell line R37C9VM6 which failed to produce primary tumours in any animals. Of the cell lines that produced primary tumours, lung metastases were detected in 32 – 71% of the tumour-bearing animals (Table 1). This range compares with the 24% lung metastases reported for the Rama 37 cells transfected with C9-Met-DNA and with no metastases from the Rama 37 cell line (Oates et al, 1996; Chen et al, 1997). There was no correlation between cellular morphology and percent metastasis (Table 1).

Osteopontin and S100A4 mRNA levels

Northern blotting showed that six of the nine clonal cell lines had significantly higher levels of the correct size, 1.6 kb, osteopontin mRNA than the Rama 37 cell line, with the increase in levels ranging from 2.8- to 11.1-fold (Student’s t-test, $P \leq 0.046$) (Table 2) (Figure 1A, C). Osteopontin mRNA levels were corrected for differences in loading/transfer by normalisation to the 28S ribosomal RNA (Materials and Methods). The remaining three cell lines R37C9VM6, R37C9VM8 and R37C9VM29 failed to exhibit significantly elevated levels compared with the Rama 37 cell line. Furthermore, with the exception of R37C9VM18, all the clonal cell lines were shown to be significantly different from the C9-Met-DNA transfant pool of Rama 37 cells ($P < 0.05$) (Table 2). When the metastatic potential in vivo and the relative osteopontin mRNA levels in the cultured cell lines were plotted against each other, there was a significant linear relationship between them (linear regression analysis: $R^2 = 0.74$, $P = 0.0007$) (Figure 1D). The mRNA levels of another metastasis-inducing gene in this system, S100A4, failed to show significant differences for either the clonal cell lines or the pooled transfectants in comparison with that of the Rama 37 cell line ($P > 0.05$) (Table 2) (Figure 1B, C). Furthermore, when the metastatic potential of the cell lines in vivo was plotted against S100A4 mRNA levels, there was no simple linear relationship between the two variables (linear regression analysis: $R^2 = 0.21$, $P = 0.16$) (Figure 1E). There was also no correlation between the levels of osteopontin mRNA and S100A4 mRNA (Table 3). The control cell line from a transplantable metastatic tumour, Rama 800 expressed both mRNAs at the correct size (Figure 1A, B).

C9-Met-DNA copy number

The number of copies of C9-Met-DNA integrated into the genome of each of the cell lines was assessed by Southern blotting. Six of the clonal cell lines from the C9-Met-DNA transfectants possessed approximately 80 – 170 copies of C9-Met-DNA fragments per haploid genome (R37C9VM3, R37C9VM8, R37C9VM13, R37C9VM14, R37C9VM18 and R37C9VM25) (Table 2) (Figure 2A). The remaining clonal cell lines possessed much lower C9-Met-DNA copy numbers of 0 – 10 (Table 2) (Figure 2A). Seven of the nine clonal cell lines had significantly higher numbers of C9-Met-DNA (Table 2).

Table 2

Relative levels of osteopontin and S100A4 mRNA and C9-Met-DNA copy number in the C9-Met-DNA transfectants

| Cell line | OPN $^a$ mRNA | S100A4 $^a$ mRNA | C9-Met-DNA $^b$ copy number |
|-----------|---------------|-----------------|----------------------------|
| R37       | 1$^e$ (±1.13) | 1 (±0.23)       | 0                          |
| R37C9VM3  | 11.1 (±0.55)$^c$ | 1.6 (±0.15)     | 147 (±17)$^c$              |
| R37 C9 VM6 | 0.5 (±0.18)$^c$ | 0.8 (±0.47)     | 0                          |
| R37 C9 VM8 | 1.4 (±0.14)$^c$ | 1.2 (±0.19)     | 169 (±8)$^c$              |
| R37 C9 VM13 | 5.5 (±0.20)$^c$ | 1.6 (±0.16)     | 133 (±10)$^c$              |
| R37 C9 VM14 | 8.0 (±0.5)$^c$ | 0.6 (±0.38)     | 95 (±6)$^c$               |
| R37 C9 VM16 | 2.8 (±0.16)$^c$ | 1.5 (±0.15)     | 8 (±3)$^c$                |
| R37 C9 VM18 | 4.5 (±0.24)$^c$ | 0.8 (±0.05)     | 83 (±5)$^c$               |
| R37 C9 VM25 | 9.3 (±1.1)$^c$ | 2.2 (±0.43)     | 145 (±17)$^c$             |
| R37 C9 VM29 | 6.7 (±0.21)$^e$ | 1.3 (±0.34)     | 3 (±0.3$^e$               |
| R37C9Pooled | 4.3 (±0.26)$^c$ | 0.8 (±0.14)     | 50$^c$                     |

$^a$Relative mRNA levels determined by Northern blotting. $^b$C9-Met-DNA copy number determined by Southern blotting. $^c$Significantly different from Rama 37 ($P < 0.05$; Student’s t-test). $^d$Significantly different from Rama 37 ($P < 0.05$; Student’s t-test). $^e$Data from Chen et al (1997). Values in brackets represent the mean ± standard deviation of three separate experiments.

Table 1

Morphology in culture and incidence of tumours and metastases in vivo of C9-Met-DNA transfectants

| Cell line | Morphology in culture | No. of rats | No. of tumour bearing rats$^a$ | No. of tumour bearing rats with metastases$^b$ | Percent metastasis$^c$ |
|-----------|-----------------------|-------------|-------------------------------|---------------------------------------------|------------------------|
| R37       | Cuboidal              | 20$^d$      | 16$^d$                        | 0$^d$                                       | 0$^d$                  |
| R37C9VM3  | Cuboidal with pseudopodia | 21          | 21                            | 15$^*$                                      | 71$^*$                 |
| R37C9VM6  | Cuboidal, tightly clustered | 20          | 0                             | 0                                           | 0                      |
| R37C9VM8  | Pseudocuboidal$^a$    | 22          | 22                            | 7$^*$                                       | 32$^*$                 |
| R37C9VM13 | Pseudocuboidal$^a$    | 21          | 21                            | 11$^*$                                      | 52$^a$                 |
| R37C9VM14 | Pseudocuboidal$^a$ with pseudopodia | 18          | 18                            | 12$^*$                                      | 67$^a$                 |
| R37C9VM16 | Cuboidal, tightly clustered | 20          | 20                            | 8$^*$                                       | 40$^a$                 |
| R37C9VM18 | Elongated with pseudopodia | 18          | 18                            | 8$^*$                                       | 44$^a$                 |
| R37C9VM25 | Elongated             | 21          | 21                            | 13$^*$                                      | 62$^a$                 |
| R37C9VM29 | Cuboidal, very tightly clustered | 18          | 18                            | 6$^*$                                       | 33$^a$                 |
| R37C9Pooled | Mixed                | 21$^h$      | 21$^h$                        | 5$^*$                                       | 24$^a$                 |

$^a$Assayed after 3 months. $^b$Determined histologically. $^c$No. of tumour-bearing rats with metastases/No. of tumour-bearing rats x 100. $^d$Data from Chen et al (1997). $^e$Statistically greater than Rama 37 ($P < 0.05$; Fisher’s exact test). $^f$Apparent absence of expression caused by over- and underlapping of elongated cellular processes (Warburton et al, 1981; Rudland et al, 1989).
Figure 1  An example of a Northern blot for OPN and S100A4 mRNA levels in the clonal cell lines. The following samples were electrophoresed through 0.8% (w/v) formaldehyde agarose gels and blotted onto Hybond-N membrane: Rama 800 (lane 1), R37C9Pooled (lane 2), R37C9VM13 (lane 3), R37C9VM16 (lane 4), R37C9VM8 (lane 5), R37C9VM16 (lane 6), R37C9VM25 (lane 7), Rama 37 (lane 8), R37C9VM29 (lane 9), R37C9VM14 (lane 10), R37C9VM3 (lane 11), R37C9VM18 (lane 12). In panel (A) the membrane was incubated with 32P-labelled S100A4 cDNA and subjected to autoradiography. In panel (B) the membrane was incubated with 32P-labelled S100A4 cDNA and subjected to autoradiography. In panel (C) the ethidium bromide-stained 18S and 28S ribosomal RNAs are shown. (D) Graph of OPN mRNA levels for the clonal cell lines in culture relative to that in Rama 37 cells (Relative OPN mRNA level) plotted against their metastatic potential in vivo (% Metastasis) (Materials and Methods). (E) S100A4 mRNA levels of the clonal cell lines in culture relative to that in Rama 37 cells (Relative S100A4 mRNA level) plotted against their metastatic potential in vivo (% Metastasis) (Materials and Methods). (F,G) Contain results for 9 C9-Met-DNA-transfected Rama 37 cell lines plus the uncloned C9-Met-DNA-transfected cells and the parental Rama 37 cells.

Table 3  Pairwise comparison of the cellular and molecular characteristics of transfectants

| Pairwise comparison* | R²,b | P⁷ |
|----------------------|-----|----|
| OPN mRNA level/plateplating efficiency | 0.91 | 5.9 x 10⁻⁶d |
| OPN mRNA level/growth potential | 0.03 | 0.63 |
| OPN mRNA level/C9-Met-DNA copy no. | 0.43 | 0.03c |
| OPN mRNA level/metastatic potential | 0.74 | 0.0007c |
| OPN mRNA level/S100A4 mRNA level | 0.16 | 0.23 |
| S100A4 mRNA level/plateplating efficiency | 0.21 | 0.16 |
| S100A4 mRNA level/growth potential | 0.01 | 0.76 |
| S100A4 mRNA level/metastatic potential | 0.21 | 0.16 |
| S100A4 mRNA level/C9-Met-DNA copy no. | 0.16 | 0.22 |
| C9-Met-DNA copy no./metastatic potential | 0.48 | 0.02c |
| Plating efficiency/metastatic potential | 0.73 | 0.0008d |
| Growth potential/metastatic potential | 0.2 | 0.17 |

*No. of cell lines tested by linear regression analysis is 11: nine clonal cell lines of the C9-Met-DNA-transfected cells plus the uncloned C9-Met-DNA-transfected Rama 37 cells and the parental Rama 37 cells. Linear regression coefficient (R²). Probability (P) that population lies on a straight line. *Significant at a 5% level.

Met-DNA copies than the Rama 37 cell line (Student’s t-test; P<0.05) (Table 2). The remaining two cell lines R37VM6 and R37C9VM29 did not have significantly higher levels. Moreover, the clonal cell lines R37C9VM3, VM8, VM13 and VM25 possessed significantly different levels of C9-Met-DNA compared to the C9-Met-DNA transfecant pool of Rama 37 cells (Student’s t-test; P<0.05) (Table 2). When the number of integrated copies of C9-Met-DNA was plotted against the metastatic potential, there was a moderate but significant correlation (linear regression analysis: R²=0.48, P=0.02) (Figure 2B) (Table 3). There was also a moderate but significant correlation between the C9-Met-DNA copy number and the level of OPN mRNA (linear regression analysis: R²=0.43, P=0.03) but not with levels of S100A4 mRNA (linear regression analysis: R²=0.16, P=0.22) (Table 3).

Growth rates

Growth curves were constructed for each cell line in order to determine their average growth rate. All the cell lines that had been transfected previously with C9-Met-DNA showed a statistically significant reduction in growth rate over that obtained for the untransfected Rama 37 cells (Student’s t-test; P<0.012). When the relative growth rates were plotted against their metastatic potential, there was no statistically significant correlation between the two variables (linear regression analysis: R²=0.2, P=0.17) (Table 3). There was also no correlation of growth rate with levels of osteopontin mRNA (R²=0.03, P=0.63) or of S100A4 mRNA (R²=0.01, P=0.76) (Table 3).
Adhesive potential

Adhesive potential of the C9-Met-DNA transfectants was determined from their plating efficiency over a 15-min period in conditioned medium exposed for 3 days to the growing transfectants (Materials and Methods). Seven of the nine C9-Met-DNA transfectants possessed a statistically increased plating efficiency over that of the untransfected Rama 37 cells (Student’s t-test; P < 0.002) (Figure 3A). The remaining two cell lines, R37C9VM6 and R37C9VM29, failed to show a significant increase. When the plating efficiency was plotted against the metastatic potential, there was a statistically significant linear correlation between these two variables (linear regression analysis: R² = 0.73, P = 0.0008) (Figure 3B). There was also a highly significant correlation of adhesive potential with levels of OPN mRNA (R² = 0.91, P = 6 × 10⁻⁶), but not with levels of S100A4 mRNA (R² = 0.21, P = 0.16) (Table 3). To test whether the conditioned medium or the cells were causing the increase in adhesiveness of the OPN-expressing transfectants, all the transfec-
tants were retested for adhesive potential in fresh routine medium not exposed to cells. There was no significant difference in plating efficiency for any of the transformant or parental cell lines when tested in routine or conditioned media (Student’s t-test, P > 0.05). Furthermore, under these conditions, there was also a statistically significant correlation between plating efficiency and metastatic potential (linear regression analysis, R² = 0.78, P = 0.00031), and plating efficiency and levels of OPN mRNA (R² = 0.93, P = 2 × 10⁻⁶) (not shown); the levels of correlation were similar to those observed earlier when transformant and parental cell lines were tested in their conditioned media rather than in fresh, unconditioned media.

DISCUSSION

To assess the association of levels of osteopontin mRNA with metastasis and other molecular and cellular characteristics, a series of single-cell-cloned cell lines has been isolated from the original pool of C9-Met-DNA Rama 37 transfectants described by Chen et al (1997). That the C9-Met-DNA-transfected clonal cell lines possess a range of morphologies in culture and show different metastatic potentials in vivo suggests that the pool of C9-Met-DNA-transfected Rama 37 cells is a mixed population of cell types. This suggestion may explain why a linear correlation between the osteopontin mRNA levels and metastatic potential of the various pooled transfectants produced from the other Met-DNAs (C6-Met-DNA, etc.), although significantly increased, was not observed (Chen et al, 1997). For example, the Rama 37 cells transfected with C6-Met-DNA induced a relatively high level of metastasis (50%), but the increase in osteopontin mRNA was relatively modest (1.6-fold), whereas Rama 37 cells transfected with C9-Met-DNA induced a lower level of metastasis (23%), but showed a relatively high increase in osteopontin mRNA (4.4-fold).

Previously, osteopontin mRNA levels have been suggested to be the effectors of all the Met-DNA fragments, including C9-Met-DNA, in the induction of metastasis (Oates et al, 1996; Chen et al, 1997). Furthermore, the levels of osteopontin mRNA have been shown to reflect the levels of osteopontin protein in Met-DNA-transfected Rama 37 cells (Oates et al, 1996; Chen et al, 1997; El-Tanani et al, 2001). Now the dose-dependent relationship observed between osteopontin mRNA levels in culture and metastatic potential in vivo for the clonal cell lines provides strong evidence that, in the case of these C9-Met-DNA-transfected clonal cell lines, metastatic potential in vivo is linearly correlated with the osteopontin mRNA levels found in the cultured cells. Hence, it is highly probable that increases in the relative amounts of osteopontin protein produced by the tumour cells is the final effector of at least C9-Met-DNA. Moreover, another gene known to induce metastasis in Rama 37 cells, that for S100A4 (Davies et al, 1993), does not have significantly higher levels of mRNA in the C9-Met-DNA clonal cell lines than in the untransfected Rama 37 cell line. Thus, it is highly likely that increased osteopontin mRNA levels (and hence protein) are the direct cause of the elevated metastatic potential in the C9-Met-DNA-transfected Rama 37 cells. This conclusion is substantiated by experiments that show overexpression of osteopontin by stable transfection of an expression vector in Rama 37 cells induces a metastatic phenotype (Oates et al, 1996). Furthermore, the association of osteopontin overexpression both with tumour progression and with metastasis has been documen-
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Bukholm IK, Nesland JM, Borresen-Dale AL (2000) Re-expression of E-cadherin at more advanced stages of malignancy, E-cadherin appears to be re-expressed (Bukholm et al, 2000). In this report, osteopontin mRNA levels are linearly correlated with cellular adhesive potential in this set of clonal C9-Met-DNA-transfected Rama 37 cell lines. This result suggests that the mechanism by which the increased osteopontin levels cause increased metastatic potential in these cells is not just increased cellular adhesion, at least in part, although other mechanisms cannot be excluded. Since there was no difference in the plating efficiency when transformant and parental cell lines were tested in their conditioned or fresh unconditioned media, the increase in adhesiveness of the transfecants arises almost exclusively from the cells themselves and not from material secreted into the conditioned medium. Increases in cellular adhesion could allow attachment of the tumour cells to vessel walls before extravasation and migration to their chosen site of metastasis. This hypothesis is supported by the evidence that osteopontin has also been shown to mediate cellular adhesion of several different types of cells including human breast cancer cells, transformed NIH 3T3 cells and erythroleukaemic cells (Liaw et al 1994; Barry et al, 2000).

Some evidence is growing to support the hypothesis that osteopontin does not only affect cellular adhesion but also affects the ability of tumour cells to metastasize. The inhibition of apoptosis by OPN has been shown in a variety of cell types including human breast cancer cells and endothelial cells (Lopez et al, 1996; Khan et al, 2002). Additionally, osteopontin-deficient vascular smooth muscle cells show increased apoptosis and decreased adhesion. This not only suggests further evidence for the role of osteopontin in cellular adhesion but also suggests that osteopontin has an inhibitory effect on metastasis (Kantar and Kramer, 1998; Day et al, 1999). This suggests that osteopontin inhibits apoptosis in metastatic cells and not just increased cellular adhesion are responsible for the cell lines with high levels of osteopontin mRNA becoming metastatic in vivo. However, the results clearly demonstrate that the C9-Met-DNA-transfected cells have not simply gained a selective growth advantage over untransfected Rama 37 cells, since the growth rates of the clonal cell lines in vitro do not correlate separately with either metastatic potential or osteopontin mRNA levels. Moreover, the benign untransfected Rama 37 cell line possesses a significantly faster growth rate in vivo than the majority of the clonal cell lines and the latent period before the tumours appear in vivo is similar in the parental Rama 37 cell line and in the C9-Met-DNA-transfected clonal cell lines, both results mitigating against growth advantage being the cause of metastasis in this system.

That there is only a moderately significant linear relationship between metastatic potential and C9-Met-DNA copy number suggests that, although the presence of C9-Met-DNA is essential, it may not necessarily be sufficient for the transfected Rama 37 cell to become metastatic in vivo. Digestion of genomic DNA by EcoR1 was complete in all cases, as described in Materials and Methods, so eliminating the possibility of abnormal numbers of integrated copies of transfected OPN cDNA being recorded. C9-Met-DNA has been postulated to act by the removal of the transcription factor Tcf-4 from the osteopontin promoter, where it acts normally to suppress osteopontin transcription (El-Tanani et al, 2001). For Tcf-4 to be removed from the osteopontin promoter by the C9-Met-DNA, the integrated copies of C9-Met-DNA in the chromatin need to be in a conformation that is accessible to proteins, that is unwound. This state would probably depend on their becoming integrated into a region of the genome of a Rama 37 cell that is actively transcribed. Different sites of integration of the con- catamers of C9-Met-DNA (Chen et al, 1997) could explain the observation in this report that occasionally large numbers of integrated C9-Met-DNA fragments occur in transfected cells with low levels of osteopontin mRNA and a low metastatic potential (e.g. R37CVM8).

To conclude, this report proves that the original pool of C9-Met-DNA-transfected Rama 37 cells is a mixed population of cells with various molecular and cellular characteristics. Moreover, the close association between osteopontin mRNA levels and metastatic potential of the C9-Met-DNA clonal cell lines provides further evidence that the direct effect of C9-Met-DNA is indeed mediated through the increase in osteopontin mRNA levels. Finally the pairwise linear correlations between metastatic potential, and both osteopontin mRNA levels and plating efficiency of the cells suggests that the mechanism by which osteopontin increases metastatic potential is mediated, at least in part, by increasing the adhesion of the tumour cells in this system.

ACKNOWLEDGEMENTS

We thank Mrs Angela Platt-Higgins and Mr Joe Carroll for assistance with histology and tumour collection, and the Cancer and Polio Research Fund for a studentship to VM and for consumables support.
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