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Biochemical responses in cultured cells following exposure to $^{89}SrCl_2$: potential relevance to the mechanism of action in pain palliation

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Abstract

$^{89}SrCl_2$ is currently used as a systemic radioactive palliative treatment for painful osseous metastases associated with an osteoblastic reaction in bone. However, the biological mechanism by which $^{89}SrCl_2$ mediates pain palliation remains unclear. In this study, attempts were made to elucidate the mechanisms by which $^{89}SrCl_2$ might influence pain at these sites. Both the direct radiotoxic effects of $^{89}SrCl_2$ on cell viability and its influence on cellular biosynthetic activity were investigated. The direct radiotoxic effects of $^{89}SrCl_2$ and X-rays were compared using the prostate carcinoma cell line, PC-3. Comparable effects upon PC-3 cell viability were seen in response to exposure to an equivalent dose given by $^{89}SrCl_2$ and X-rays (2 Gy). Experiments to investigate the indirect action of $^{89}SrCl_2$ exposure employed the MC3T3-E1 cell line and focused on their production of Prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6). Exposure of the MC3T3-E1 cell line to $^{89}SrCl_2$ resulted in an increased production of PGE_2 in a concentration-dependent manner. No increased PGE_2 production was seen by the MC3T3-E1 cells in response to X-ray exposure either in the presence or absence of $SrCl_2$. IL-6 was produced by the MC3T3-E1 cells in response to $^{89}SrCl_2$ exposure via a PGE_2-mediated pathway. This study demonstrates the release of potent biochemical modifiers of bone turnover in response to the systemically applied radiotherapeutic $^{89}SrCl_2$. This strongly suggests the mechanism of pain palliation by $^{89}SrCl_2$ is likely to result from a complex interaction of direct and indirect radiation-induced effects.

Keywords: Radiopharmaceutical; Strontium; Radioisotope; Prostaglandin; E_2; Interleukin-6; Pain palliation; Bone

1. Introduction

Metastatic bone disease is associated with the progression of many different tumour types and is one of the most common causes of pain in cancer patients [1]. $^{89}SrCl_2$ is currently used as a palliative treatment for painful osseous metastases associated with an osteoblastic reaction in bone. This would include those arising from both prostate and breast carcinoma [2]. $^{89}SrCl_2$ has been shown to be selectively taken up and retained at sites of bone metastases [3,4]. However, the mechanism by which $^{89}SrCl_2$ provides pain relief at these sites remains poorly understood.

It has been suggested that $^{89}SrCl_2$ acts as a calcium mimic [5,6] and accumulates at sites of osteoblastic lesions through incorporation into the mineralising collagen during new bone formation. A recent *in vitro* study demonstrated $^{89}SrCl_2$ accumulation to occur in the presence of the differentiated osteoblast-like cell line, MC3T3-E1. In this study, localisation of the $^{89}SrCl_2$ was observed to be extracellular and occur during the mineralisation of newly synthesised collagen [7]: This would account for $^{89}SrCl_2$ accumulation at sites of new bone formation. Although the role of $^{89}SrCl_2$ in pain palliation is not clear, accumulation of a radiation dose at such sites, via incorporation of $^{89}SrCl_2$, could potentially affect a number of cell populations in close proximity. These would include metastatic tumour cells, as well as local bone cell populations. Such cells are likely to undergo radiation-induced changes primarily affecting their viability, but also effecting biosynthetic activity. Some clinical observations appear to support a role for tumour cell kill in providing pain relief [8]. Others, however, have shown pain relief to be independent of tumour radiosensitivity indicating that the

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palliative response may not be entirely a result of radiotoxicity [9]. Therefore, it may be considered that the occurrence of secondary radiation-induced changes to the tumour and surrounding bone cell population are likely to have a key role in pain palliation and possibly tumour viability.

This study examines the effect of $^{89}SrCl_2$ on both cell viability and the production of cellular factors that may influence pain production at the lesion. This has included an investigation into the levels of prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) produced in response to $^{89}SrCl_2$ exposure as both have been shown to have major roles in influencing bone remodelling [10,11].

2. Materials and methods

2.1. Reagents

All tissue culture reagents were purchased from Gibco BRL, with the exception of L-glutamine, penicillin and streptomycin, which were purchased from ICN Biomedicals. $^{89}SrCl_2$ and [*methyl*-^{14}C]thymidine were obtained from Amersham Pharmacia Biotech. All other reagents were purchased from Sigma.

2.2. Cell culture

MC3T3-E1 cells were purchased from the Riken cell bank, Japan, and cultured in alpha-modified Eagle's medium (a-MEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50 μg/ml). PC-3 cells were purchased from ECCAC and cultured in Nutrient Ham's F-12 medium containing 10% FBS, 2 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50 μg/ml). All cell incubations were at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. X-ray irradiation of cell cultures

PC-3 cells were seeded into 25 cm^2 tissue culture flasks (500 cells/flask) approximately 4 h prior to irradiation. Flasks were irradiated with a 250 kVp X-ray machine operated at 240 kV and 15 mA. Absorbed doses were calculated using appropriate temperature and pressure conversion factors. The dose rate given was approximately 0.6 Gy/min. Colonies were allowed to develop at 37 °C for 10–14 days prior to fixing and staining in 2% crystal violet in 95% methanol. Colonies greater than 50 cells were scored.

2.4. $^{89}SrCl_2$ irradiation of cell cultures

Cells were seeded into 25 cm^2 tissue culture flasks (625 cells/flask) in a total of 5 ml of media approximately 6 h prior to exposure to $^{89}SrCl_2$. To irradiate the cells, aliquots of aqueous $^{89}SrCl_2$ were added to duplicate flasks to give a range of $^{89}SrCl_2$ concentrations. After 24 h, media was removed from each flask and cells washed twice with phosphate-buffered saline (PBS) to remove residual radioactivity. Fresh growth media was returned to each flask and colonies were allowed to develop at 37 °C for 10–14 days. Colonies were fixed and stained in 2% crystal violet in 95% methanol. Colonies greater than 50 cells were scored.

2.5. $^{89}SrCl_2$ induced PGE_2 and IL-6 production

MC3T3-E1 cells were seeded into 24-well tissue culture plates at 1.25×10^4 per well in a total volume of 0.5 ml. After 4 days incubation, media was removed from all wells and replaced with fresh growth media (0.5 ml). Varying amounts of aqueous $^{89}SrCl_2$ were added to replicate wells to give a range of $^{89}SrCl_2$ concentrations up to 100 μCi/ml. Indomethacin was added to duplicate wells at each $^{89}SrCl_2$ concentration to give a final concentration of 3 μM. Incubations were allowed to continue for 7 days at which point media was collected from each well and stored at −20 °C for future analysis of the PGE_2 and IL-6 content.

2.6. X-ray induced PGE_2 and IL-6 production

MC3T3-E1 cells were seeded into 25 cm^2 flasks at a concentration of 2.5×10^4 cells/ml in a total of 5 ml growth media. After 4 days growth, media was removed from each flask and replaced with growth media containing a range of $SrCl_2$ concentrations. Flasks were irradiated with X-rays to give a total dose of 2, 4 and 6 Gy. Appropriate sham-irradiated controls were performed. Flasks were further incubated at 37 °C for 24 h after which a sample of media was removed from each flask and stored at −20 °C until it was assayed for PGE_2 and IL-6.

2.7. PGE_2, IL-6 and IL-β determination

Frozen MC3T3-E1 media samples were allowed to thaw at room temperature. Samples were centrifuged at 10 000 *g* for 30 s to pellet any cell debris. PGE_2, IL-6 and interleukin-1β (IL-1β) content of each of the sample were determined using enzyme-linked immunosorbent assay kits purchased from Amersham Pharmacia Biotech. Protocols were followed as described by the manufacturer. Growth media was used as the standard and sample diluent in the IL-6 assay.

2.8. Cell viability

MC3T3-E1 and PC-3 cells were seeded into 96-well culture plates at a density of 2×10^3 cells/well. PGE_2 was

added to wells after 24 h to give a concentration range of 0.975 ng/ml to 17.5 μg/ml. Ethanol was used as the vehicle control. Cells were incubated for a further 6 days prior to the incubation for 7 h with [*methyl*-^{14}C]thymidine (0.1 μCi/well). Incorporated radioactivity was isolated by precipitation of the cell monolayer in the culture wells with the addition of 10% (w/v) ice cold trichloroacetic acid (TCA). The TCA soluble fraction was removed and the remaining insoluble fraction washed twice with ice-cold ethanol. Removal of the TCA insoluble fraction from the wells was with 0.1 M NaOH. Radioactive content was determined by liquid scintillation counting.

3. Results

A series of cell-based studies have been undertaken which were designed to elucidate the mechanism by which $^{89}SrCl_2$ exerts radiation-induced changes upon cell viability and biosynthetic activity of cells in close proximity. Such experiments employed the MC3T3-E1 (murine pre-osteoblast) and the PC-3 (human prostate adenocarcinoma) cell lines.

Initial experiments concentrated on investigating the direct radiotoxic effects of $^{89}SrCl_2$ on a tumour cell population. In addition, a comparison was made between the degree of radiotoxicity observed as a result of $^{89}SrCl_2$ and external beam irradiation. The PC-3 cell-line was employed for use in toxicity assays as it has been well characterised with regards to its radiosensitivity [12]. Experiments compared the viability of the PC-3 cell line after exposure to a total dose of 2 Gy given by external beam irradiation or $^{89}SrCl_2$ present in the cell culture media (Fig. 1). The mean percentage survival of the PC-3 cell line at a total dose of 2 Gy was 40% (23–54%) given by X-rays and 38% (22–54%) given by incubation with $^{89}SrCl_2$. This compares with a previous literature report for PC-3 survival at 2 Gy of 32% (25–38%).

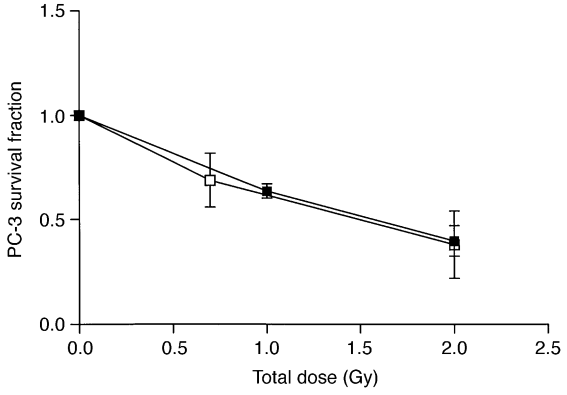

Fig. 1. PC-3 cell survival upon exposure to X-rays or $^{89}SrCl_2$. Cells were exposed to either X-rays, $n=4$ (solid) or $^{89}SrCl_2$ (20–100 μCi/ml) $n=2$ (open). Error bars represent standard error of the mean (S.E.M.).

Further experiments attempted to identify other non-lethal cellular events that may be occurring in response to $^{89}SrCl_2$ exposure. Of particular interest were changes in cellular events that might play a role in bone remodelling and also in pain production. The pain-associated factor, PGE_2 has been demonstrated to also be a potent modulator of bone remodelling. In addition, PGE_2, was also shown to influence both cell proliferation and cellular radiosensitivity. Therefore, experiments examined changes in the production of PGE_2 in response to both $^{89}SrCl_2$ and X-ray exposure. The study employed the MC3T3-E1 cell line, a cell line that has previously been shown to produce both PGE_2 and IL-6 *in vitro* [13]. Experiments demonstrated a concentration-dependent increase in PGE_2 production on exposure to $^{89}SrCl_2$ (Fig. 2). A maximum PGE_2 production of 11 ng/ml was observed at the highest $^{89}SrCl_2$ concentration (100 μCi/ml). This represented a 14-fold increase in the PGE_2 production compared with cells not exposed to $^{89}SrCl_2$. A timecourse demonstrated increased PGE_2 production to occur within 2 h after exposure with the maximum production being achieved after 24 h exposure to $^{89}SrCl_2$ (Fig. 3).

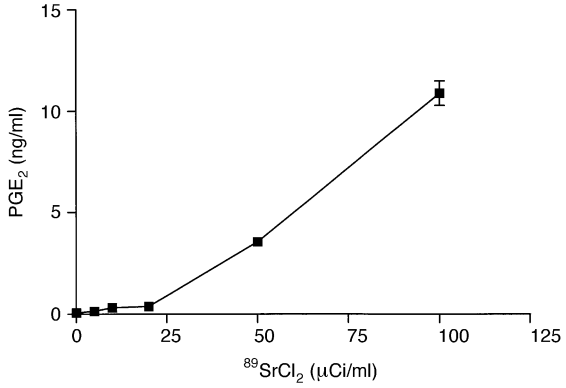

Fig. 2. Prostaglandin E_2 (PGE_2) production by MC3T3-E1 cells upon exposure to an increasing concentration of $^{89}SrCl_2$ (0–100 μCi/ml). Error bars represent the standard error of the mean (S.E.M.) ($n=2$).

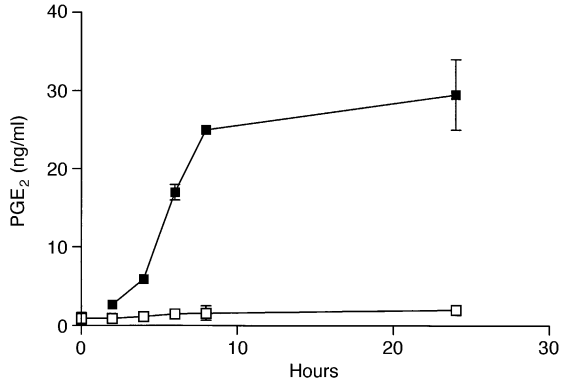

Fig. 3. Timecourse of Prostaglandin E_2 (PGE_2) production by MC3T3-E1 cells upon exposure to $^{89}SrCl_2$. MC3T3-E1 cells were incubated in the presence (solid) or absence (open) of $^{89}SrCl_2$ (50 μCi/ml). Error bars represent standard error of the mean (S.E.M.) ($n=2$).

The study investigated whether PGE_2 production was purely in response to radiation or whether $SrCl_2$ itself had a role in the increased levels of PGE_2 observed. MC3T3-E1 cells were irradiated with X-rays (0–6 Gy) in the presence and absence of $SrCl_2$. The results demonstrated there to be no increased production of PGE_2, over basal levels, in response to X-rays alone (data not shown). However, PGE_2 production was seen to be elevated in the presence of increasing concentrations of $SrCl_2$ alone. PGE_2 production in cells exposed to 6 mg/ml $SrCl_2$ was demonstrated to increase by 13-fold above cells incubated without $SrCl_2$ (Fig. 4). Subsequent experiments investigated the production of PGE_2 when increasing quantities of $SrCl_2$ were added to a fixed radioactive concentration of $^{89}SrCl_2$. Results demonstrated PGE_2 production in the presence of $^{89}SrCl_2$ to be typically 1.5-fold greater than with $SrCl_2$ alone, despite the overall chemical amounts of $SrCl_2$ being equivalent (Fig. 5).

How increased PGE_2 production might effect local cell viability was also considered. Changes in PC-3 and MC3T3-E1 cell viability in response to PGE_2 was determined by [*methyl*-^{14}C]thymidine incorporation. Results demonstrated that PGE_2 at concentrations seen to be produced by the MC3T3-E1 cells (250 ng/ml), had no significant effect on cell viability. However, reduced cell viability was seen for both of the cell lines when exposed to concentrations of PGE_2 greater than 5 μg/ml (Fig. 6a and b). Incorporated [*methyl*-^{14}C]thymidine was reduced by 83 and 81% for the MC3T3-E1 and PC-3 cells, respectively, when incubated in the presence of the maximum PGE_2 concentration studied (17.8 μg/ml).

PGE_2 has been shown to influence IL-6 production by MC3T3-E1 cells [13]. Therefore the MC3T3-E1 media samples collected from the above experiments were also analysed for the presence of IL-6. Analysis of the samples demonstrated the production of IL-6 by the MC3T3-E1 cells occurs in response to both $SrCl_2$ and $^{89}SrCl_2$ with a maximum mean production of 100 pg/ml in the presence of $^{89}SrCl_2$ (50 μCi/ml). However, an equivalent concentration of $SrCl_2$ only resulted in 25 pg/ml IL-6 being produced by an equal number of the MC3T3-E1 cells. In addition, IL-6 production was shown to be completely inhibited by the cyclooxygenase inhibitor indomethacin (Fig. 7) indicating IL-6 production to be via a PGE_2-mediated pathway. IL-1β was not detected in any of the samples analysed (data not shown).

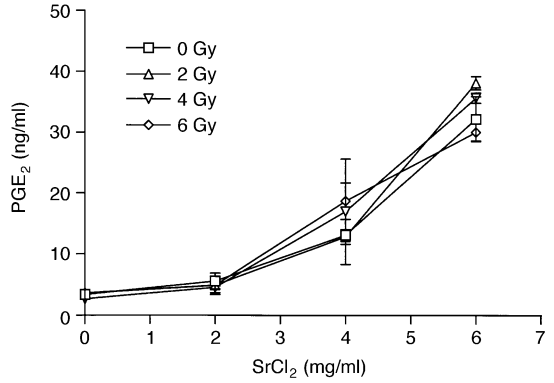

Fig. 4. Prostaglandin E_2 (PGE_2) production by MC3T3-E1 cells upon exposure to X-rays in the presence of $SrCl_2$. Media was collected 24 h post-irradiation. Error bars represent standard error of the mean (S.E.M.) ($n=2$).

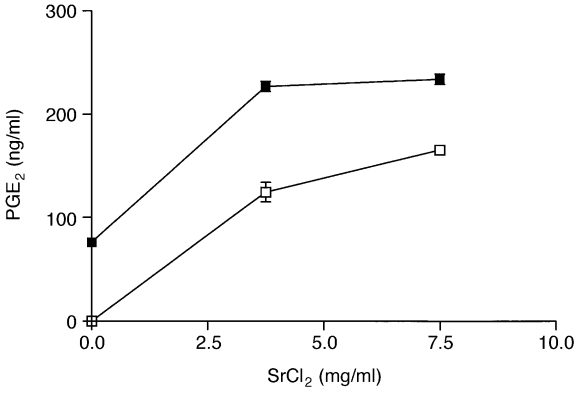

Fig. 5. Prostaglandin E_2 (PGE_2) production by MC3T3-E1 cells upon exposure to $^{89}SrCl_2$ (50 μCi/ml)/$SrCl_2$. Error bars represent standard error of the mean (S.E.M.) ($n=2$). ■, represents use of $^{89}SrCl_2/SrCl_2$; □, represents use of $SrCl_2$ only.

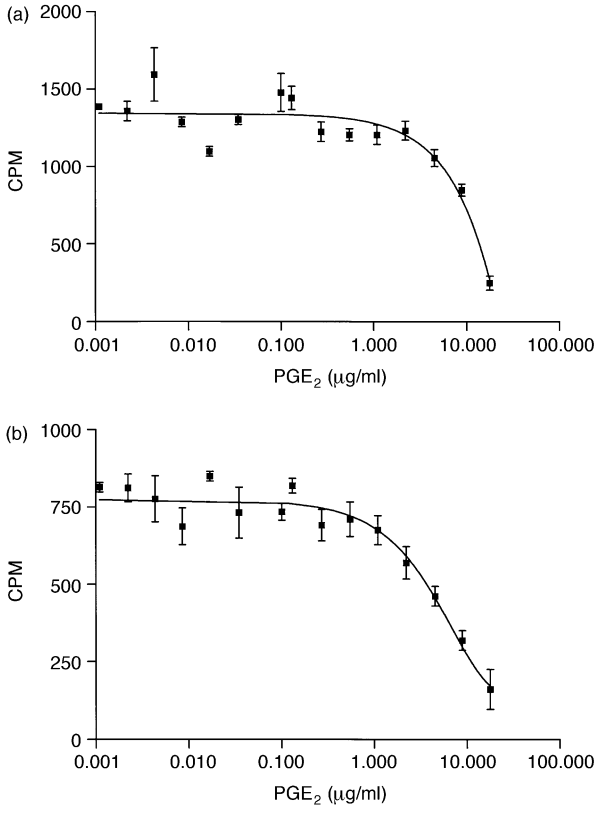

Fig. 6. Effect of Prostaglandin E_2 (PGE_2) on (a) MC3T3-E1 and (b) PC-3 cell viability. Cells seeded at 2000 cell/well were incubated for 24 h prior to incubation with [^{14}C-*methyl*]thymidine. Error bars represent standard error of the mean (S.E.M.) ($n=3$). cpm, counts per minute.

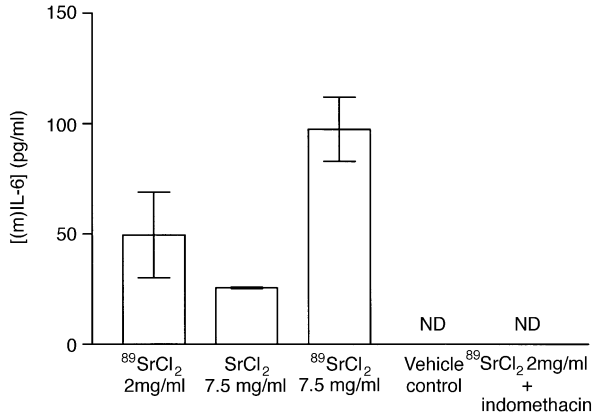

Fig. 7. Interleukin 6 (IL-6) production by the MC3T3-E1 cells upon exposure to $^{89}SrCl_2/SrCl_2$. Error bars represent standard error of the mean (S.E.M.) ($n=2$). ND, not detected.

4. Discussion

The initial part of this study investigated the direct radiotoxic effects of $^{89}SrCl_2$. The results presented demonstrate $^{89}SrCl_2$ exposure to cause reduced PC-3 cell viability. In addition, the degree of radiotoxicity was seen to be comparable to that resulting from an external radiation source at an equivalent total dose of 2 Gy. $^{89}SrCl_2$ is a high energy (1.492 MeV) beta-emitting nuclide with a half life of 50.4 days. It is evident that once $^{89}SrCl_2$ has accumulated at the site of the metastatic lesion its retention is very high, possibly remaining in the bone indefinitely [14]. Studies to calculate the absorbed radiation dose by the metastatic tumour from $^{89}SrCl_2$ by direct means have concluded that the dose to the tumour ranges from 1.3 to 64 Gy, (mean 18 ± 16 Gy) [8]. An absorbed dose of this magnitude is likely to produce radiological effects leading to reduced viability of cells associated with the tumour [15,16]. In bone metastases, however, the impact on the different cell types associated with the lesion, osteoblasts and osteoclasts and the ensuing palliative effects, are less well defined.

In addition to investigating the direct effects of $^{89}SrCl_2$ in a cell population, this study also demonstrates $^{89}SrCl_2$ to cause indirect effects on the biosynthetic activity of an osteoblast-like cell line. The data presented above demonstrates the pre-osteoblast cell line, MC3T3-E1 to produce elevated levels of PGE_2 in response to exposure to $^{89}SrCl_2$, with an initial stimulation of PGE_2 production occurring within 2 h of exposure. As a potent modulator of bone remodelling [17,18], PGE_2 stimulates the replication of the pre-osteoblast [19] and shows biphasic effects on both DNA synthesis and alkaline phosphatase production [20]. The role of PGE_2 in bone resorption is likely to be mediated through the synthesis of IL-6 by osteoblasts, which is regulated by PGE_2 intracellular signalling [13]. PGE_2 has been also been implicated as a mediator of tissue inflammation [21]. Therefore it may be considered that $^{89}SrCl_2$ induced PGE_2 production may potentially influence both local pain production and bone remodelling. It has been noted that upon administration of $^{89}SrCl_2$, patients have been reported to experience a sudden increase in pain shortly afterwards. This has been termed the 'flare response'. Increased pain at the lesion site could be attributed to increased inflammation in response to the initiation of local PGE_2 production. In addition to causing increased inflammatory pain, the significance of an increasing level of PGE_2 site might be in influencing bone formation as PGE_2 concentrations lower than that required to stimulate resorption have been shown to increase collagen synthesis [18,22] and stimulate alkaline phosphatase activity [20,23]. $^{89}SrCl_2$ has been demonstrated to accumulate during mineralisation of newly synthesised Type I collagen [7]. Therefore, increased collagen synthesis by PGE_2 as a consequence of the initial localisation of $^{89}SrCl_2$, may be beneficial in facilitating increased $^{89}SrCl_2$ localisation. Subsequently, PGE_2 was shown to cause a reduction in MC3T3-E1 and PC-3 cell viability at concentrations of around 5 μg/ml. Previous studies have demonstrated PGE_2 to have an effect on both viability and radiosensitivity of a number of cell types. Although results from *in vitro* studies are conflicting, PG-induced radiation protection has consistently been demonstrated *in vivo* [24,25]. The blocking of the arachidonic acid cascade with cyclooxygenase inhibitors and the use of PGE_2 receptor site antagonists has been considered in an attempt to overcome PGE_2 production and the resulting radioprotection [26]. However, in the case of the $^{89}SrCl_2$ mechanism of action, the production of PGE_2 at the lesion site in response to exposure may be desirable as PGE_2 production may positively influence the outcome of the treatment through its influence on bone biology.

This study demonstrates production of IL-6 by the MC-3T3-E1 cell line in response to $^{89}SrCl_2$. Enhanced production of IL-6 was inhibited by indomethacin and therefore appears to have been produced via a PGE_2-dependent pathway at PGE_2-concentrations greater than 1 μM (Fig. 7). This is in agreement with previous studies that demonstrated PGE_2 to significantly stimulate IL-6 secretion in a dose-dependent manner between 1 nM and 10 μM in the MC3T3-E1 cell line [13]. IL-6 is a multifunctional cytokine, of which circulating levels are below the limits of detection under physiological conditions [27]. In addition, IL-6 has been demonstrated to be a potent stimulator of bone resorption by osteoclasts [10]. Therefore, it may be considered that increased IL-6 production at the lesion site may stimulate local activation of osteoclasts resulting in enhanced bone resorption at a site that has previously displayed overactive bone formation. The consequence of this

may be a restoration of the coupling process of bone remodelling leading to reduced local bone formation. However, the levels of PGE_2 and IL-6 reported in this study cannot easily be related to those produced *in vivo* or indeed, to a concentration required to effect osteoclast resorption *in vivo*. Local concentrations of PGE_2 and IL-6 have not as yet been quantified.

In summary, this study demonstrates the release of chemical modifiers, namely PGE_2 and IL-6, in response to the systemically applied radiotherapeutic, $^{89}SrCl_2$. The results support the intriguing possibility that the effectiveness of $^{89}SrCl_2$ as a palliative agent in the treatment of bone metastases, may be due to a combination of both direct and indirect effects; these being the direct radiotoxic effect of $^{89}SrCl_2$ on the tumour and bone cell viability and, additionally, its ability to indirectly induce the production of PGE_2 and IL-6, both of which have significant roles in bone biology.

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