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The cytotoxic effect of methotrexate loaded bone cement on osteosarcoma cell lines

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Abstract We mixed various amounts of methotrexate with bone cement and measured the absorbance daily for 4 weeks. The cytotoxic effects on SaOS2 and MG63 osteosarcoma cells were examined by the MTT assay, and analysed according to the methotrexate concentration and the elapsed time. The amount of eluted methotrexate was greatest during the first day, and then decreased rapidly reaching a plateau in the third week. The number of viable tumour cells decreased significantly after 72 h, and they were hardly seen after 1 week.

Résumé Nous avons étudiés le mélange de methotrexate avec du ciment à os à des concentrations variables et mesuré la diffusion journalière pendant quatre semaines. L'effet cytotoxique sur les cellules ostéosarcomateuses SaOS2 et MG63 a été examinés par l'essai MTT, et analysé d'après la concentration du méthotrexate et le temps écoulé. Le montant de méthotrexate relargué était plus grand pendant le premier jour, et a ensuite diminué pour arriver à un plateau dans la troisième semaine. Le nombre de cellules tumorales viables a diminué considérablement après 72 heures pour être presque nul après une semaine.

Introduction

Bone cement has almost no effect on the proliferation of osteoblast-like cells in vitro [5], and for this reason it has been used as a vehicle for local drug delivery, and appropriate concentrations of drugs in the required area have been maintained for long periods of time. There are many reports of satisfactory results in the treatment of chronic osteomyelitis and in post-surgical infections using this method of delivering drugs [2, 4, 8, 13].

Maintaining high concentrations of chemotherapeutic agents is particularly important in the treatment of primary malignant bone tumours. The biocompatibility of cement allows adjuvant local chemotherapy treatment in the area of surgery by incorporating the drugs into the cement. There have been a few reports [7, 14, 15, 16] of this form of treatment, with no reports of its use in osteosarcoma, which is the commonest primary malignant bone tumour, and one that requires high concentrations of chemotherapeutic drugs. We believe that the successful development of this mode of delivery of drugs will prove to be of considerable use in the treatment of bone tumours. We chose methotrexate (MTX) for this study, as it is a commonly used chemotherapeutic agent.

Materials and methods

Preparation of MTX loaded bone cement pellets

Pellets containing MTX were prepared using methotrexate powder (Choongwae Pharmacy) and Simplex P bone cement (Howmedica, USA), which is composed of 40 g of polymethylmethacrylate powder (including 4 g of barium sulphate) and 20 ml of solvent (2.6% of polymerisation accelerator *N*,*N*-dimethyl-*p*-toluidine and 97.4% of methylmethacrylate monomer). The pellets were prepared by mixing 5, 10, 15, 20 and 50 mg of methotrexate powder with 50 mg of cement and 0.25 ml of polymer solution. Each pellet was 12 mm in diameter and 2 mm thick.

The measurement of MTX elution from MTX loaded cement pellets

The hardened pellets were rinsed for 1 min in flowing distilled water sterilized with 70% ethanol for 20 min and rinsed with distilled water again for 2 min. The pellets containing different amounts of methotrexate were placed in five wells of a six-well plate, and a pellet which did not contain methotrexate was placed

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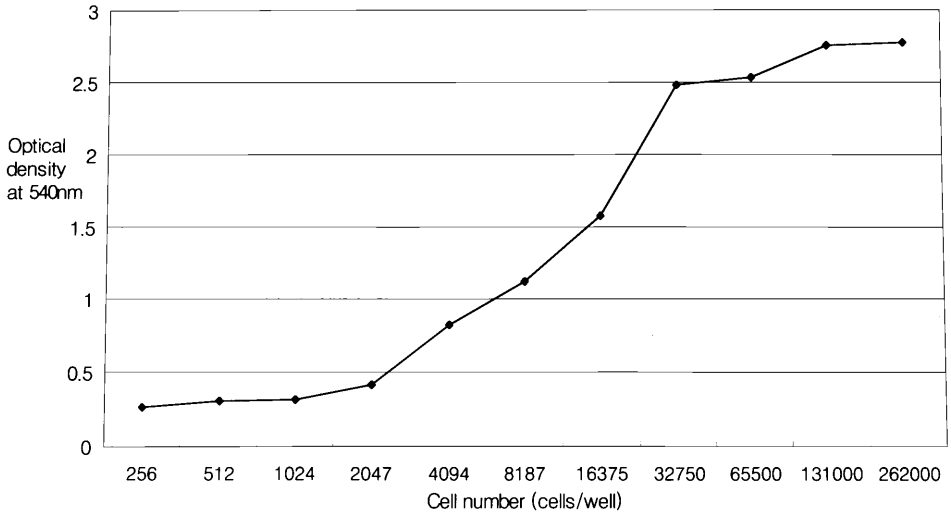

Fig. 1 The growth curve of the MG63 cells. The appropriate number of cells for the MTT assay was 1.6×10^4 cells/well in the log phase of the growth curve

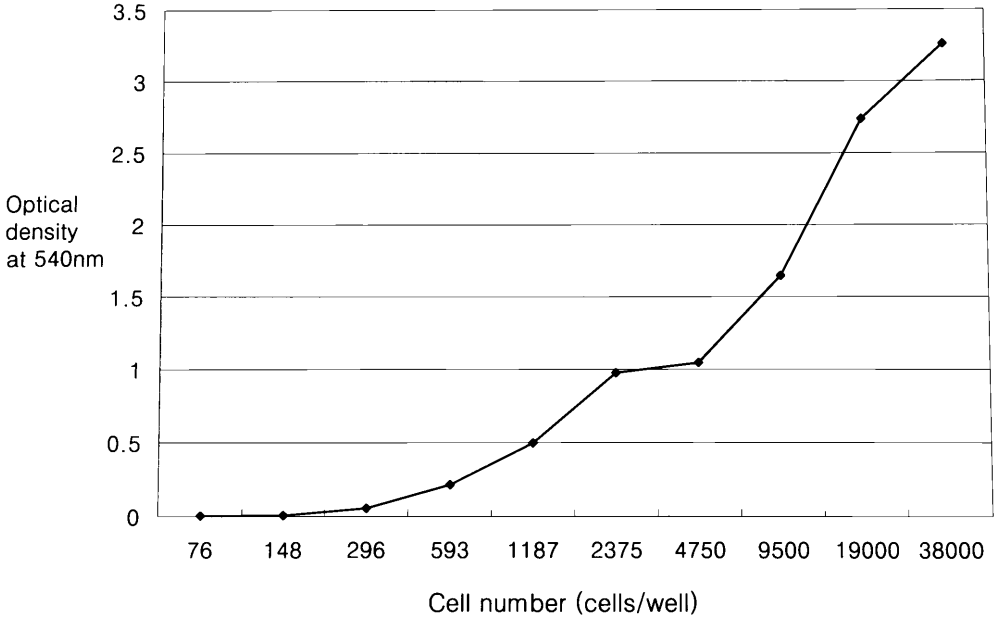

Fig. 2 The growth curve of SaOS2 cells. The appropriate number of cells for the MTT assay was 9.5×10^3 cells/well in the log phase of the growth curve

in the remaining well as a control for spectrophotometric analysis. All wells contained 5 ml of HBSS (Hanks' balanced salt solution). The well plate was incubated at 37°C in a 5% CO_2 atmosphere. Concentrations of eluted methotrexate in HBSS were measured daily for 4 weeks using a spectrophotometer (DU 650; Beckman, Fulton, Calif., USA) at 302 nm. Five millilitres of HBSS was removed daily for the measurement and replaced again, in order to measure the amount eluted after every 24 h.

Cultures of osteosarcoma cell lines and cytotoxicity assay of eluted MTX on osteosarcoma cells

Two kinds of human osteosarcoma cell lines (from Korean Cell Line Bank, College of Medicine, Seoul National University, Seoul, Korea) were used – SAOS2 and MG63 cell lines from primary osteosarcoma. The rates of growth did not change with time and the cells maintained the characteristics of osteosarcoma after sequential cultures. SAOS2 cells were cultured in a 75-cm^2 flask containing RPMI 1640 medium (Gibco, Grand Island, N.Y., USA), and MG63 cells were grown in a flask containing DMEM medium (Dulbecco's modified Eagle's medium; Gibco). Each medium included 10% foetal bovine serum (FBS; Gibco), 100 units/ml of penicillin G sodium and 100 units/ml of streptomycin sulphate. Osteosarcoma cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

The frozen cell lines were rapidly warmed in a 37°C water bath and diluted in culture medium. After 5-min centrifugation at 1500 rpm, the upper part of the solution was removed and the remaining cells placed in a culture flask (75 cm^2).

Determination of appropriate cell numbers for cytotoxicity assay

When MG63 cells reached 80–90% cellular confluence, the fault culture cells were separated with trypsin-EDTA (Gibco, Grand Island, N.Y., USA). The separated cells were diluted in DMEM medium and centrifuged for 5 min at 1500 rpm. The upper part of the solution was removed, the cells were resuspended in DMEM medium and cell numbers were counted using a hemocytometer. One-hundred microlitres of the suspension containing 2.6×10 cells was placed in the first well of a 96-well plate and then 100 µl of medium was added. Cell concentration was diluted by a factor of 2 in each of the subsequent wells, until the 11th well, which con-

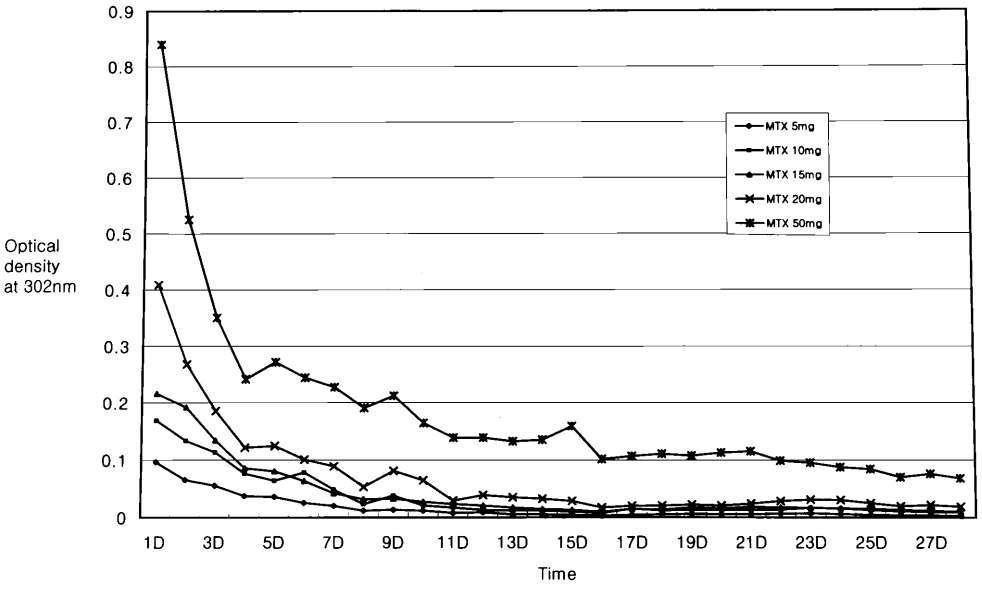

Fig. 3 Sequential changes of MTX elution from pellets according to the amount of loaded MTX. The eluted quantity of MTX was greatest during the first day, and then the amount decreased rapidly until the end of the first week, reaching a plateau in the third week

tained 256 cells. The 12th well contained 200 µl of medium only. The well plate (maintained at 4°C) was incubated for 48 h and the MTT assay, with 3-(4,5-dimethylthiazole-2-yl-2,5-diphenyltetrazolium bromide (Sigma Chemical, St. Louis, Mo., USA), diluted to 5 mg/ml with PBS (phosphate buffered saline), was used to determine the number of viable cells. Fifty microlitres of MTT was added to all wells and the well-plate incubated for a further 4 h to deoxidize MTT under light-blocking conditions. After incubation, the upper part of the solution was removed leaving 30 µl in all wells. One-hundred and fifty microlitres of DMSO (Amresco, USA) was added to each well and the well-plate stirred for 5 min. The absorbance was measured at 540 nm in an ELISA reader and the growth curve of the MG63 cells was obtained. We used 1.6×10^4 cells on the log phase of the growth curve (Fig. 1). For SaOs2 cells, the appropriate number of cells was determined to 9.5×10^5 in the same manner (Fig. 2).

Cytotoxicity assay using MTT method for MTX loaded bone cement for osteosarcoma cell lines

The appropriate numbers of cells were placed in each well of a 24-well plate. Neither methotrexate nor cement was included in the first column and only cement added to the second. From the third to the sixth column, cement pellets with differing amounts of methotrexate were included. The amounts of methotrexate used were determined according to preliminary experiments, as the cytotoxic concentrations were different for SaOS2 and MG63 cell lines. Pellets were prepared by mixing 0.01, 0.1, 1 and 10 mg of methotrexate with 50 mg of cement in the SaOS2 cell line and 0.1, 1 and 10 mg of methotrexate with 50 mg of cement in the MG63 cell lines. Five-well plates of each cell line were prepared in the same manner by culturing for 2 weeks. The numbers of viable cells in each well were measured by MTT cytotoxicity assay on the first, third, fifth, seventh and 14th days. The experiments were repeated three times respectively in SaOS2 and MG63 cell lines using the same procedures.

Statistical analysis

The cytotoxicity assay results were analysed statistically using the Kruskal-Wallis test to determine their relationship with methotrexate concentrations over time.

Results

Elution of methotrexate from the cement pellets

The sequential changes in drug elution from the pellets with differing amounts of added MTX are shown in Fig. 3. The elution was greatest during the first day for all concentrations, decreased rapidly during the next 3 or 4 days and achieved a constant rate of release after 2 weeks. The eluted methotrexate correlated with the amount incorporated into the mixture as the surface areas were the same and the amounts eluted were in direct proportion to the amount of MTX used from the 2-week stage onwards.

The proportion of the total drug eluted to the amount incorporated during the first week was a minimum (5.4%) for the MTX 15-mg pellet and a maximum (6.8%) for the MTX 10-mg pellet. The mean amount of methotrexate eluted from the different pellets was 6.1% for the first week and 9.6% (7.2–11.7%) over the 4-week period.

The actual concentration of methotrexate was 4.24×10^{-5} M on the first day and 1.36×10^{-6} M on the 28th day with the MTX 5-mg pellet. These concentrations were 130 to 4000 times higher than the minimum inhibitory concentration (MIC, 1×10^{-8} M) required to inhibit DNA synthesis [3]. In the case of the MTX 20-mg and 50-mg pellets, the concentrations of methotrexate were 10,000 times higher than the MIC on the first day and 1400 times higher on the 28th day.

Cytotoxicity assay of eluted MTX on the osteosarcoma cells

Results of MTT assay on SaOS2 cell lines

Figure 4 shows the inhibition of the growth of SaOS2 cell lines by the eluted MTX using the MTT assay. The

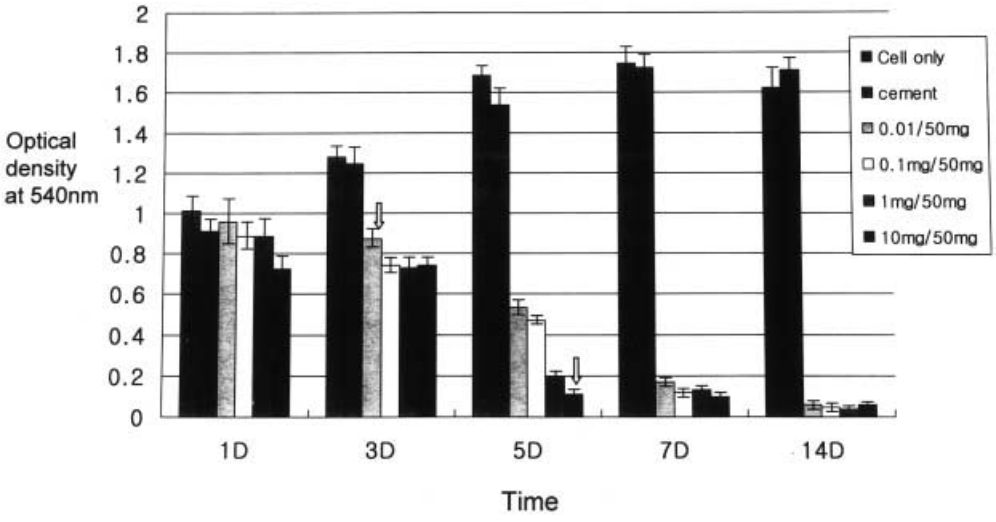

Fig. 4 The result of the MTT assay of SaOS2 cells. A decrease in the number of viable tumor cells was significant after 3 days of culture. The culture revealed a significant decrease in the number of viable cells after 3 days in the group containing the smallest amount of MTX (0.01 mg; *P*=0.004, *black arrow*). In the group containing the largest quantity of MTX (10 mg), the decrease was significant after 1 day and remained more prominent 5 days after culture (*P*=0.002, *white arrow*)

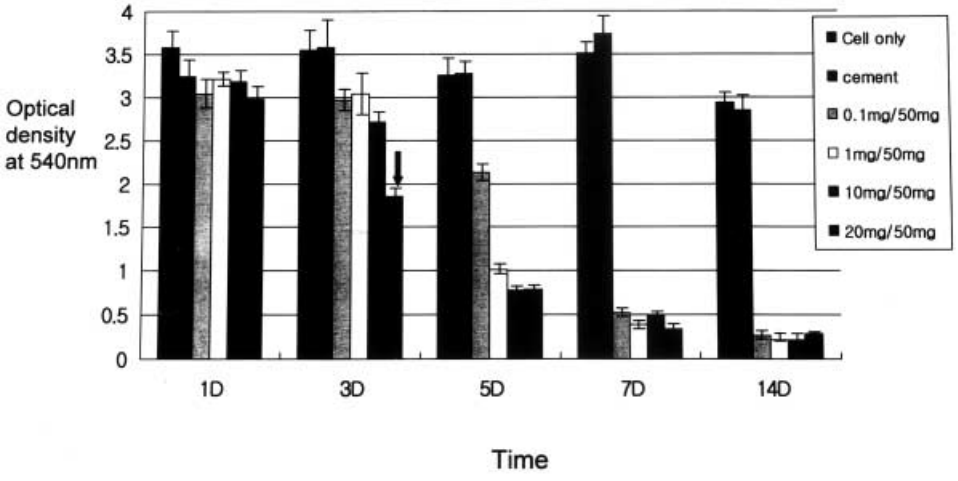

Fig. 5 The result of the MTT assay of MG 63 cells. Cells cultured with cement pellets containing 20 mg of MTX showed a significant decrease on the third day, and this was most prominent in the group containing the largest amount of MTX (*P*=0.008, *arrow*). Viable cells were hardly observed in all of the four groups containing MTX loaded cement pellets after 1 week

control group (cells only) and the cement group (cement without MTX) showed that the number of cells increased until the fifth day and then plateaued. These groups were similar to each other and the cement did not affect cellular proliferation (*P*=0.5).

All the MTX groups showed that the number of viable cells decreased significantly from the third day (*P*=0.004) and that viable cells were hardly seen on the 14th day. The MTX 0.01-mg group showed no difference from the control group on the first day, but the difference was evident from the third day and the number of viable cells decreased progressively until the 14th day (*P*=0.004). In the MTX 10-mg group there was a significant decrease in the number of viable cells from the first day and a rapid decrease between the third and fifth days (*P*=0.002).

Results of MTT assay on MG63 cell lines

Cell numbers in the control (and cement only) groups initially increased, reaching a plateau after 7 days, and decreased slightly on the 14th day. The number of cells decreased from the third day in the four MTX groups; this was most evident in the MTX 20-mg group (*P*=0.008 vs. the control). With the exception of the MTX 0.1-mg group, decreasing numbers of cells in the other MTX groups became more apparent after the fifth day, and very few viable cells were seen on the 14th day (Fig. 5).

Discussion

Hernigou et al. [7] first reported that the local concentration of a chemotherapeutic agent was maintained at a high level in malignant tumours in dogs. Kirchen et al. [11] reported that MTX loaded cement could reduce the incidence of recurrence in the treatment of giant cell tumour. Greco et al. [6] suggested that adriamycin and cisplatin eluted from cement may be effective against colon and breast tumour cells. In our study, methotrexate eluted from cement inhibited the proliferation of SaOS2 and MG63 cell lines, and the biological activity of methotrexate was preserved. Wang et al. [14] showed that methotrexate dissolved at 185–204°C and lost its phar-

maceutical characteristics at this temperature. Thus the moderate heat generated during the polymerization of cement (70°C) exerted no detrimental effect on methotrexate. However, adriamycin and vincristine lose their pharmaceutical characteristics at temperatures exceeding 95°C. The deleterious effects of the polymerization itself as well as of the heat generated should be borne in mind. Tetracycline and chloramphenicol for example are inactivated by polymerization. In our study we were able to show that methotrexate was stable during the polymerization of cement.

The mechanism of elution of the drugs from the cement has not been determined. Several experiments support the hypothesis that the drugs diffuse across a concentration gradient and are eluted through the pores in the cement [10, 13]. Therefore, a higher local concentration of the drug can be maintained by increasing the surface area of cement. In the treatment of osteomyelitis, antibiotic loaded beads of cement have been used. However, affecting the shape of the cement is almost impossible in tumour surgery, and we may have to depend on the amount of drug incorporated into the cement to control the level of its release.

We did not compare different kinds of cement using only simplex (Surgical Simplex P bone cement; Howmedica, USA); however, it is likely that different types of cement will also affect the local concentration of eluted drugs. With regard to antibiotics, mixing 2 g with 40 g of cement has been shown to have no effect on the strength of this cement [9, 12]; and Wang et al. [14] also reported that methotrexate had almost no effect on the mechanical characteristics of cement at a dose of 2 g to 40 g mix of drug to cement.

It would be more advantageous if we could achieve an adequate antitumour effect with a minimal quantity of eluted drug. In the four concentrations used in this study, the concentrations of methotrexate were 100 to 10,000 times higher than the minimum inhibitory concentration (MIC, 1×10^{-8} M) required to inhibit DNA synthesis and this was maintained over a 4-week period. Variable rates of drug elution have been reported; Hoff et al. [8] reported 5% elution over an 8-week period, and Wroblewski et al. [17] reported that 78% of antibiotic was retained in cement after 2 years. Wasserlauf et al. [16] showed that 6% of antitumour drug was eluted during the first 6 months. The elution rate is primarily related to the nature and the surface area of the cement, in addition to the effect of the concentration. In our study the mean amount of methotrexate which was eluted was 6.1% during the first week and in total 9.6% was eluted during the first 4 weeks; the amounts which were eluted were in proportion to the amount of drug in the cement. This was observed consistently during the 4-week study period; and thus the elution rate was related to the concentration gradient.

MTT assay has been widely used to evaluate cellular susceptibility not only to antitumour drugs and growth factors but also for other biological materials. The assay is simply performed with small amounts of cells and medium, and many different materials can be examined in a short time. It is a valuable way of estimating the effects of diverse drugs, including antitumour drugs, and the toxicity of new materials. The cells are usually cultured in a 96-well plate and the absorbance measured using ELISA.

In our study, the cells were cultured in a 96-well plate in order to determine the number of cells. In order to evaluate the cytotoxicity of the eluted methotrexate, culture using the 3-mm pellets was not possible in a 96-well plate. Instead the cells were cultured in a 24-well plate, on five occasions, and the absorbance of each well was measured using a spectrophotometer. However, the number of cells in each well was the same as that of the 96-well plate. The medium was not exchanged during the study period in order to accumulate the eluted drug. The numbers of viable cells did not decrease until the end of the study period, in the case of the control and cement groups; and thus we can assert that the reduction in the number of viable cells in MTX loaded cement was caused by the accumulated drugs and not by the lack of medium.

The number of viable cells was similar in the control (cells only) and cement groups (cement without MTX) in both cell lines, but the decreasing numbers of cells differed in a time and concentration dependent manner in the MTX containing groups. There also appeared to be differences between the two cell lines chosen with regard to their susceptibility to the chemotherapeutic agents. In both cell lines, the differences in the number of viable cells became evident on the third day and viable cells were only rarely seen after the first week.

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