Preclinical development of biodegradable polymer foils for intracerebral delivery of cytotoxic nucleosides

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Intracerebral implantation of biodegradable polymers loaded with cytotoxic or radiosensitising nucleoside analogues is a promising treatment strategy for malignant gliomas, which are currently intractable. The aim of the study was to develop biodegradable polymers containing nucleosides which could be implanted intracerebrally. Methods of synthesis were developed for the copolymers composed of D,L-lactide, glycolide and caprolactone in different proportions, as well as a novel method of introducing nucleosides to these copolymers at the polymerisation step. Upon degradation in an aqueous medium some of these copolymers emit nucleosides in micromolar concentration over several months. Their in situ degradation and biocompatibility with brain tissues was assessed by means of scanning and transmission electron microscopy. At the ultrastructural level tissue responses to the copolymer implantation closely resembled the responses to mechanical trauma.

key words: biodegradable polymers, biocompatibility, brain tissues, electron microscopy

INTRODUCTION

Biodegradable polymeric carriers can be used for the local delivery of pharmaceutically active substances [7]. In particular, biodegradable polymers containing cytotoxic drugs are being developed for the treatment of primary malignant brain tumours, anaplastic gliomas and glioblastoma multiforme [3]. This therapeutic approach may be especially well suited to overcome the main limitation that seems to preclude the development of efficacious systemic glioma treatment, i.e. peripheral toxic effects which occur at drug doses below those required to establish the cytotoxic concentration of a drug in the vicinity of malignant cells located behind the blood-brain and blood-tumour barrier.

Several nucleoside analogues are cytotoxic toward glioma cells and/or provide substantial radiosensitisation in vitro and in animal glioma models. These include, inter alia, 5-fluorouracil (5-FU) [26], gemcitabine (dFdC, 2',2'-difluoro-2'-deoxycytidine) [22, 25], tezacitabine (FMdC, (E)-2'-deoxy-2'-(fluoromethylene)-cytidine) [23, 28], cladribine (2CdA, 2-chloro-2'-deoxyadenosine) [5, 11, 19], given systemically bromodeoxyuridine (BrUdR) and iododeoxyuridine (IudR) [14]. However, the nucleoside analogues tested to date, including the newer gemcitabine and cladribine [10, 24], are clinically inactive in anaplastic gliomas.

In contrast, the intracerebral application of 5-FU-containing biodegradable microspheres for the radiosensitisation of glioblastoma in a pilot non-randomised trial appeared to almost double median survival time compared to the historical control [21]. This last result is, to
our knowledge, the first clinical proof of the efficacy of malignant glioma treatment by intracerebral implantation of a nucleoside analogue-containing biodegradable copolymer.

In the present paper we summarise our studies concerning the preclinical development of novel biodegradable polymers based on lactide, glycolide and/or caprolactone for intracerebral delivery of cytotoxic nucleosides.

**MATERIAL AND METHODS**

Novel non-toxic initiators of polymerisation were employed to produce various biodegradable block copolymers composed of D,L-lactide, caprolactone and/or glycolide in various proportions. The aforementioned polymerisation initiators included suitable lithium, magnesium, and zirconium compounds (such as zirconium [IV] acetylacetonate, or dibuthylmagnesium). The details of the copolymer syntheses and their physico-chemical characteristics have been described elsewhere [8, 15].

Nucleosides (a “model” nucleoside adenosine, cytotoxic nucleosides cladribine and 5-FU, or a radiosensitising nucleoside bromodeoxyuridine) were introduced to the copolymers during the polymerisation process, either by mechanical mixing (i.e. in the form of powders), or after dissolving them with a suitable organic solvent (such as hexafluoroisopropanol). The nucleoside content in the polymers was 5% (w/w). After precipitation with methanol and drying, the polymers were formulated into either the microspheres (diameter 10–60 μm), or polymer “foils” (thickness 0.04–0.08 mm). After air-drying, these formulations were subjected to sterilisation, either by exposure to ethylene oxide, or by gamma irradiation. The *in vitro* assessment of the rate of release of nucleosides from the copolymer foils was performed using an intermittent perfusion system, described in detail in the previous papers [16, 17]. The influence of the sterilisation procedure on the *in vitro* rate of release of nucleosides was also evaluated for the chosen polymers.

Animal experiments were conducted according to the procedures reviewed and approved by the Ethical Committee of the Polish Academy of Sciences Medical Research Centre. To assess biocompatibility with brain tissues the polymers were implanted into the brains of male Wistar rats (200–250 g body weight), which were kept under transient anaesthesia with a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg). The skull above the temporal lobes was exposed by skin incision, and holes in the bone (3 or 6 mm in diameter, for microsphere injection and polymer strip insertion, respectively) were made bilaterally with the use of a dental drill, followed by incision of the dura and electrocoagulation of the small brain surface arteries to avoid bleeding. Subsequently, depending of the formulation of the test polymer to be used, one of the following procedures was applied. Polymer microspheres were mixed with 10 μl of physiological saline to obtain 20% suspension, which was stereotactically injected into the temporal lobe using a Narishige micromanipulator and a 25-gauge needle. The injection was performed slowly (within 2 mins.) to avoid gross mechanical trauma of the brain tissues. The equivalent volume of the solvent was injected into the opposite temporal lobe. In the second procedure a piece of temporal lobe tissue was bilaterally removed with the use of a 2 mm diameter surgical vacuum aspirator inserted to the depth of 3–4 mm. A strip of a polymer under study was inserted in place of the removed tissue fragment with the use of a small forceps, and on the contralateral side a similar strip of a commercially available sterile gelatine sponge (Spongostan, Ferrosan, Denmark) was inserted. Finally, dental acrylic resin was used to plug the holes and the skin was sutured. The aforementioned procedures were performed in sterile conditions, and postsurgically the animals were treated by ceftriaxon (Biotraxon, Bioton) 50 mg/kg/day for one week to prevent infection.

In order to evaluate *in situ* copolymer degradation and brain tissue response patterns following the insertion of polymer strips, transmission and scanning electron microscopy were employed. At different times after the polymer implantation (ranging from one day to 12 weeks) the animals were anaesthetised as described above and perfused transcardially with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.5% cacodylate buffer (pH 7.4). Tissue processing for transmission EM was performed as previously described in detail [31]. For scanning EM the procedure described in detail by Groniowski et al. [12] was used with some modification, including drying of the tissue material at the critical point. Micrographs were obtained with a JEM 1200EX electron microscope and a scanning imaging device.

**RESULTS**

The data concerning the *in vitro* kinetics of the release of nucleosides from nucleoside-containing copolymers have been reported in detail elsewhere [16, 17]. The overall impression from these studies was that when nucleosides were introduced to the copolymers by mechanical mixing, the rate of release was not stable over a longer period, and some preparations unloaded nucleosides completely over a few days. Solubilisation of
nucleosides prior to their introduction to copolymers at the polymerisation stage resulted in a marked improvement in uniformity in the release of these substances over time.

Scanning electron microscopy enabled the surface of the trace left by the needle used to inject the copolymer microspheres to be inspected. On day three this surface was covered with the microspheres (Fig. 1) but these disintegrated relatively quickly. Three weeks after implantation their remnants were barely visible (Fig. 2). In contrast, solid fragments of the inserted copolymer strips were clearly visible in brain parenchyma as long as six weeks after the implantation (Fig. 3).

Transmission electron microscopy revealed no appreciable differences between ultrastructural features of brain parenchyma in the vicinity of the inserted reference material (Spongostan) and the polymer under study. On the third and seventh days after the insertion of the polymer strips typical ultrastructural features of tissue response to a mechanical trauma were seen, including active phagocytes with differentiated phagolysosomes (Fig. 4), proliferating microglia (Fig. 5) with phagocytic features (Fig. 6), a slight enlargement of the space between the endothelium and the basal membrane (Fig. 7), and neurocytes rich in Golgi complex and endoplasmatic reticulum (Fig. 6). At six and twelve weeks after the copolymer implantation the ultrastructural features of the blood microvessels returned to normal (Fig. 8), while some signs of astrocyte activation (Figs. 8, 9), and multiplication of Golgi complexes in neurocytes (Fig. 10) were still present.

**DISCUSSION**

The biocompatibility of biodegradable copolymeric microspheres composed of poly(D,L-lactide-co-glycolide) (PLG) [9] and poly(epsilon-caprolactone), ethylcellulose or polystyrene [20], and of polyanhydride/poly-[bis(p-carboxyphenoxy)propane-sebacic acid] (PCPP-SA) strips [4, 29] with animal brain tissues have been studied with the use of light microscopy histological and histochemical techniques. These studies have shown that the polymeric materials are well tolerated following implantation into the CNS and that the astrocytic responses are largely a consequence of the mechanical trauma, which occurs during microsphere injection or surgery. However, injecting slowly degrading microspheres to the brain parenchyma may lead to the obstruction of the medial ventricle and the development of hydrocephalus [20].
Figure 2. Remnant of disintegrating copolymer microspheres in brain parenchyma — three weeks after their implantation into the brain. Scanning EM, mag. × 8,000.

Figure 3. A fragment of the disintegrating copolymer strip in the brain parenchyma — six weeks after implantation into the postsurgical cavity. Scanning EM, mag. × 6,000.
Figure 4. Phagocyte with differentiated phagolysosomes loaded with polymer degradation products — three days after copolymer microsphere implantation. Transmission EM, mag. × 8,000.

Figure 5. Two microglial cells (M), one of them dividing (arrow) in the perivascular space — seven days after copolymer microsphere implantation. Transmission EM, mag. × 8,000.
Figure 6. Neurone (N) rich in Golgi complex and endoplasmatic reticulum, and a microglial cell with phagocytic features (M) attached to it. The vicinity of a degrading copolymer strip — seven days after implantation. Transmission EM, mag. × 8,000.

Figure 7. Blood microvessel with an enlarged space between the endothelium and the basement membrane covering a pericyte (P) and a phagocyte (F) rich in phagolysosomes in perivascular space. The vicinity of a degrading copolymer strip — seven days after implantation. Transmission EM, mag. × 8,000.
Figure 8. Precapillary vessel tighted with a pericyte, and an astrocyte with glial filaments. The vicinity of a degrading copolymer strip — six weeks after implantation. Transmission EM, mag. × 8,000.

Figure 9. An astrocyte in the perivascular space. In the perinuclear zone glial fibres are visible. Neuropil and synaptic endings are ultrastructurally unchanged. The vicinity of a degrading copolymer strip six weeks after implantation. Transmission EM, mag. × 8,000.
Recently Veziers et al. [30] have employed scanning and transmission electron microscopy to study PLG microspheres in brain tissue at the ultrastructural level after intrastriatal implantation for up to two months post-implantation. In this last study the progressive degradation of the microspheres was visualised and appeared to be independent of the presence of macrophages.

Our present results are compatible with the aforementioned reports. They show that even large strips of the copolymer foils composed of various combinations of D,L-lactide, caprolactone and/or glycolide are also well tolerated by brain tissues. Ultrastructural tissue responses were not different from those characteristic of unspecific brain injury. Importantly, our relatively large polymer foil strips did not produce brain tissue responses appreciably different from those which occur after implantation of a commercially available gelatine sponge which is believed to be fully brain-biocompatible and widely used in neurosurgery.

There are two prerequisites of the efficacy of a biodegradable polymeric preparation containing a cytotoxic drug for malignant glioma treatment. One is the requirement of an adequate concentration profile of a drug across the brain tissues surrounding the tumour focus, while the other is the requirement of a prolonged release of the compound in concentrations sufficient to kill neoplastic cells. Malignant gliomas are characterised by infiltrative growth and neoplastic cells sometimes spread to areas far distant from the tumour focus [18]. However, 90% of glioma recurrences appear within 2 cm of the contrast-enhancing tumour border [13], and the majority of glioma patients die as a result of tumour recurrence close to its original location [1]. Enhancing the distance of drug penetration across the brain may only be obtained by increasing the concentration of the drug at the tissue-polymer border, which will be limited by its toxicity toward normal brain cells. Fortunately, from the clinical point of view this may not be of primary importance, at least in the majority of cases. Prolonged exposure, in contrast, may be of critical importance, in particular in the case of drugs preferentially affecting cells during the S-phase of the cell cycle (i.e. during DNA replication), such as several cytotoxic nucleoside analogues. Halogenodeoxyuridine labelling experiments employing histochemical or PET detection of these tracers bound to DNA (Blasberg et al. [2], and the references cited) have shown that at any given time only a few percent of glioma cells are in the S phase. It should also be noted that, in the primary cultures of glioblastoma multiforme, most of the cells entering the S phase appeared...
destined to leave the cell cycle and these were continuously replaced by the quiescent clonogenic cells which started to replicate DNA [11].

Microspheres containing cytotoxic or radiosensitising drugs may be particularly useful for the treatment of surgically-inaccessible brain tumours, as they can be injected directly into the tissues surrounding the tumour focus, which may allow inoperable tumours to be reached. However, although the rate of decomposition of copolymer microspheres can be controlled by their composition and their full degradation may even take years, most of the drug-loaded microspheres tested to date decompose much faster than our copolymer strips.

With the use of our experimental set-up we were not able to measure the kinetics of release of nucleosides from microsphere formulations, but the kinetics reported by others for various cytotoxic drugs are in reasonable agreement with our scanning electron microscopy observations of the in situ microsphere degradation. Moreover, the release of drugs from drug-containing microspheres may not be uniform over time. For example, in vitro drug release from the injectable carboplatin-loaded poly(D,L-lactic-co-glycolic) acid copolymer (PLGA) microspheres developed by Chen and Lu [6] started with a 10-day slow release period, which then switched to a fast, near zero-order release period ending at 22 days.

For the reasons mentioned above, the prolonged emission of a cytotoxic drug may be a prerequisite for long-term control of brain gliomas. The camptotecin-containing microspheres developed recently by Storm et al. [27], which in vitro were able to release the drug intact for up to 1000 h (41 days), were able to prolong the median survival of rats with brain-implanted 9L-gliosarcoma from 17 days (for BCNU-treated animals serving as controls) to 69 days. One may speculate that continued prolongation of the intracerebral drug delivery period could further increase survival or even provide a cure.

Biodegradable polymer wafers or foil strips seem to erode more slowly compared to most drug-containing microsphere preparations. They may therefore ensure a greater duration of cytotoxic drug concentrations in the brain tissues. For example, the 3 × 2 mm discs of a poly(bis(p-carboxyphenoxy)-propane):sebacic acid (ratio 20:80) polymer containing tirapazamine (a hypoxia-activated DNA-damaging agent) produced by Yuan et al. [32], when incubated in PBS at 37 degrees C, showed continuous in vitro drug release for over 100 days. This result is in good agreement with our scanning electron microscopy observations of the in situ degradation of the polymer strips, remnants of which were still present in brain tissues 6 and 12 weeks after implantation. At the same time, point tissue responses to the implanted polymers closely resembled the sequela of mechanical trauma. Although the development of reactive gliosis around a polymer strip inserted into brain tissue (which may potentially interfere with drug diffusion across brain tissues) is of some concern, polymer strips may be better suited to use in multimodal glioma treatment, in which a polymer loaded with a cytotoxic or radiosensitising compound would be used postsurgically as an adjunct to tumour resection.

For copolymers loaded with nucleosides, the use of an appropriate organic solvent to dissolve a nucleoside prior to introduction to a polymer at the polymerisation stage may result in a long-term and stable emission of the nucleoside during copolymer disintegration.

ACKNOWLEDGEMENTS

This work was supported by the State Committee for Scientific Research (KBN, Poland) grant No. 405F02412. The authors thank Drs. M. Bero, J. Kasperczyk and P. Dobrzyński (Institute of Polymer Chemistry, Polish Academy of Sciences, Zabrze) for the synthesis of copolymers loaded with nucleosides. The participation of Dr Tomasz Kryczka in the in vitro assays of the kinetics of release of nucleosides from polymers and Dr Janusz Jagielski and Mr. Marek Stefanowicz in the animal experiments is acknowledged.

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