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The Construction of a Functional Photoactivatable Cancer Targeting Bispecific Antibody Conjugate

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Given their excellent potential, obtaining therapeutic antibodies which can efficiently target tumour cells has proved to be very difficult.[1] The large majority of antibodies raised against tumour antigens, even those which are quite specific for certain tumours, have some specific or nonspecific cross reactions with normal tissues.[2] This seriously restricts their use in tumour targeting. Photoactivatable antibodies[3] and antibody conjugates could be employed to solve this problem. An antibody which becomes active, only where it is required, on illumination with UV light, would make targeting much more regionally specific. This beneficial effect could be maximised by utilising bispecific antibodies.[4] In these a tumour-specific antibody is coupled to a second antibody capable of binding a substance which is directly or indirectly cytotoxic. If only the cytotoxic end of the antibody were to be reversibly-inactivated, then the tumour-specific end would remain free to bind to its target tumour cells without untoward damage to peripheral normal tissues. Localised illumination of the tumour-targeted bispecific antibody would then maximise tumour destruction whilst minimising damage to other tissues.

Herein, we describe the construction of such a bispecific conjugate. An antitumour (carcinoembryonic antigen [CEA] specific) antibody coupled to a photoactivatable monoclonal antibody which binds the enzyme alkaline phosphatase (AP). An anti-AP was chosen as AP has been suggested as a possible enzyme for use in cancer targeting,[5] is easy to detect, and thereby simplifies the analysis of the anti-AP antibody conjugates.

The anti-AP antibody was first reversibly deactivated with a 1-(2-nitrophenyl)ethanol (NPE) coating.[2] An average of ten NPE residues coated each anti-AP antibody molecule. When tested in an ELISA the NPE coated anti-AP had approx 30% of the activity of the uncoated antibody, however this increased to approximately 75% of the native value after 10 min irradiation with UV-A light from a hand held 6W UV-lamp. The NPE coupling and uncoupling reactions are well documented and the reaction schemes are given in two previous publications.[6] A major advantage of this procedure is that this low level of light does not damage the antibody or cells (see below). Many previous 'caging' procedures require either organic solutions and/or very high levels of irradiation for reactivation. Both of which are very damaging to biological molecules and cells.[7]

The NPE coated antibody was then added to AP coated sepharose beads to remove the 30% of the antibody which remained active. After absorption, the ability of the coated antibody to bind AP reduced markedly to as little as 0.5% of its uncoated value (Table 1) with between 50 and 87% of the initial monoclonal antibody activity recoverable after UV irradiation.

Table 1. The photoactivation of the NPE coated anti-AP monoclonal.[a]

UV light	0 min	5 min	10 min
12 ng/well NPE-antibody	2.9	77	87
6 ng/well NPE-antibody	<0.5	40	50
control antibody	100	116	103

[a] The values given for antibody-AP binding represent the absorbance value at 492 nm given by each sample expressed as a percentage of the absorbance value at 492 nm given by control uncoupled unirradiated antibody samples. All control and irradiated samples were measured in quadruplicate in wells of an AP-coated ELISA plate.

The anti-CEA antibody was then coupled to the NPE coated anti-AP antibody using the bifunctional crosslinker, N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP). SPDP was added to each antibody, coupling via its *N*-hydroxysuccinimide ester to antibody amine residues. After reduction of the SPDP derivatised anti-CEA conjugate to form free sulphydryl residues, it was added to the SPDP derivatised NPE-anti-AP to enable the formation of anti-CEA–anti-AP bispecific conjugates via disulphide bridges. A simplified reaction sequence is given in Scheme 1 below. For the sake of completeness this scheme also shows the uncloaking of the bispecific antibody conjugate antibody when it is bound to a tumour cell and illuminated by UV light.

When anti-CEA antibody, SPDP-derivatised anti-CEA and this anti-CEA–anti-AP bispecific conjugate were added to the wells of a CEA coated ELISA plate, followed by the addition of an AP-conjugated-anti-mouse IgG, all three antibody preparations bound to the plate in a dose dependent manner. This simple control experiment demonstrated that the derivatisation and coupling procedures had very little effect on the activity of the anti-CEA antibody. Light mediated targeting of AP to CEA coated ELISA plates was then demonstrated by repeating the above experiment but adding AP as the final detection layer. No colour was obtained with the anti-CEA, SPDP-derivatised anti-CEA, or bispecific anti-CEA–anti-AP conjugates. However AP was found to bind to wells to which UV-irradiated bispecific conjugate had been added. Human LS174T colonic carcinoma cells were then used as a target as they were known to express large amounts of CEA.[8] In a first experiment (Figure 1) the bispecific conjugate was irradiated in quartz cuvettes for 6 min,

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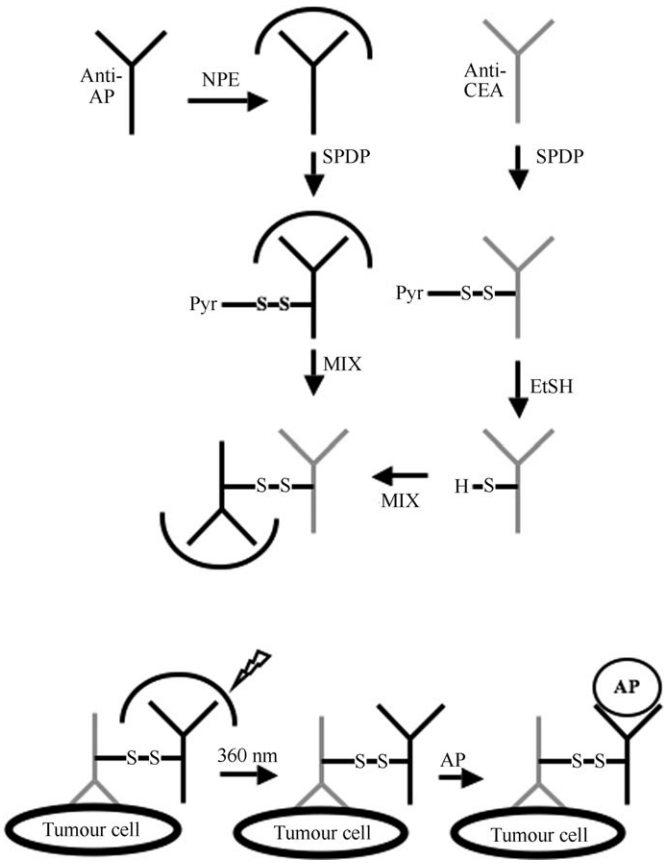

Scheme 1. A simplified reaction scheme of the steps involved in the formation of the NPE "caged" bispecific antibody conjugate and how it can be used to target a cancer cell.

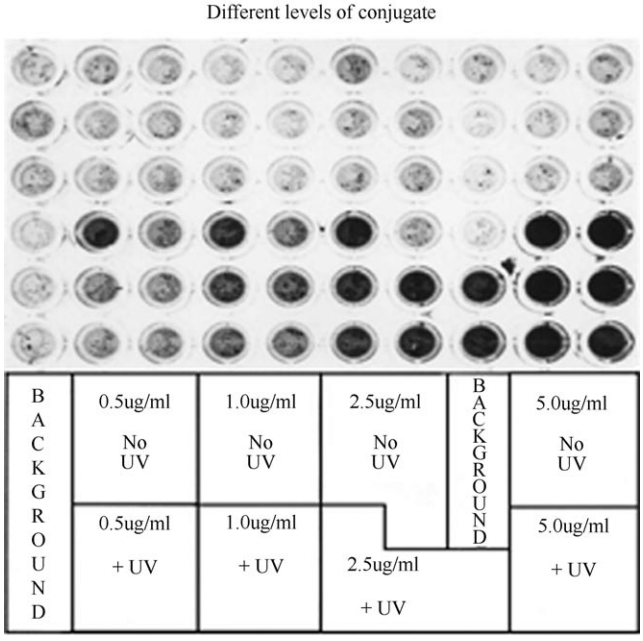

Figure 1. AP activity targeted to the surface of colon carcinoma cells by UV irradiation. A template demonstrating how much conjugate was added to which well is given directly below the plate. The outside wells of the plate were not used to eliminate edge effects.

diluted to four different concentrations, and added to a LS174T coated plate.

Cells which had been incubated with unirradiated conjugate followed by AP, bound very little AP, well OD values (0.6) being only slightly greater than those in control wells (0.5). However wells into which irradiated conjugate had been placed contained much more AP. This effect was at its clearest in wells incubated with 5 $\mu g\,mL^{-1}$ conjugate (OD 2.0) but could still be seen in wells which had only received 0.5 $\mu g\,mL^{-1}$ (OD 0.84). The LS174T cells had bound the bispecific conjugate but were only able to target AP to the cells if the conjugate was activated by UV-light.

A second experiment was then carried out to examine if the conjugate could be activated in situ with the conjugate irradiated (in the presence of the cells and media) from directly below the plate. LS174T cells were again grown to confluence in two 96 well plates with the bispecific conjugate being added at 2.5 $\mu g\,mL^{-1}$ to the top 5 rows of one plate and at 5 $\mu g\,mL^{-1}$ to the other plate. Groups of 2 or 3 columns on each plate were then irradiated for varying lengths of time by sliding the plates across the surface of the lamp. After incubation and washing, AP was added to 3 of these rows (Figure 2).

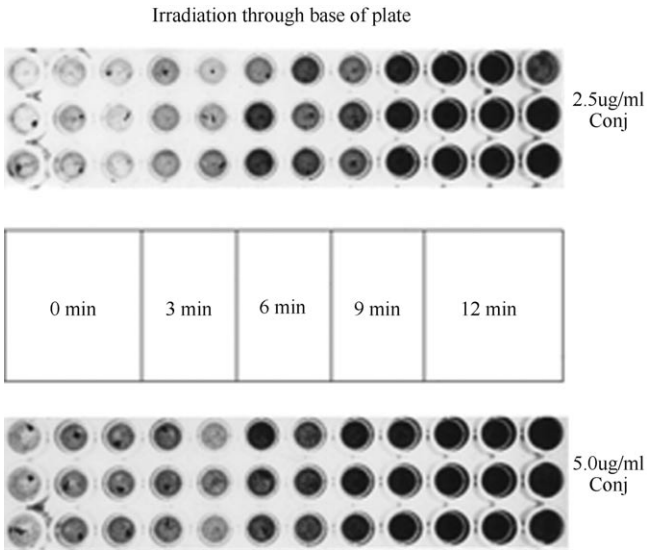

Figure 2. The in situ targeting of AP to LS174T cancer cells. Only the middle three rows of each plate are shown, as the five control rows (two rows conjugate no AP, three rows AP no conjugate) gave essentially the same background value as the unirradiated wells

More AP was targeted to the cancer cells as the time of irradiation increased, the activity of the conjugate still increasing after 12 min of UV treatment because of the plastic plate absorbing around a third of the UV-A light.

In conclusion, we suggest that use of photoactivatable bispecific antibodies would be a major step forward in tumour targeting. With activity restricted to illuminated cancer bearing areas, many anticancer antibodies previously discarded because they were not specific enough in vivo, could be transformed into useful therapeutic agents.

Experimental Section

3 mL of the anti-AP monoclonal antibody (Zymed labs, 0.25 mg mL^{-1} g mL^{-1} in 0.1 M $NaHCO_3$ pH 8.3) was inactivated by the addition of 20 μL NPE-carbonylchloride in dioxan.[2] After dialysis and centrifugation the NPE coated-anti-AP antibody (0.18 mg mL^{-1}) was obtained as a clear solution. On absorption, the concentration of the NPE-coated antibody reduced to 0.11 mg mL^{-1}.

Measurement of NPE coated anti-AP antibody activity in an ELISA: The amount of NPE-coated-anti-AP capable of binding to AP coated ELISA plates was quantitated by the addition of Horseradish peroxidase-anti-MouseIgG followed by colour development at 492 nm using *o*-phenyldiamine as substrate.

The CEA specific antibody T84.66, was concentrated from serum free medium by $(NH_4)_2SO_4$ precipitation. T84.66 reacts strongly with colonic cancer tissue and some other carcinomas but only weakly with normal tissues.[9]

Construction of the photoactivatable anti-CEA–anti-AP bispecific conjugate: The anti-CEA and NPE-coated anti-AP antibodies (each at 0.18 mg mL^{-1}) were derivatised by the addition of a 60-fold molar excess of SPDP. The SPDP–anti-CEA antibody was reduced with 0.5 M Dithiothreitol (DTT, 30 min) and dialysed to remove excess DTT. This reduced SPDP–anti-CEA was added to the unreduced SPDP-derivatised NPE–anti-AP antibody and they were left to crosslink. During the formation of the bispecific conjugates, we discovered that much better conjugation at higher yields was obtained if the NPE coated anti-AP antibody was not preabsorbed prior to addition of the SPDP.

The ability of T84.66–anti-AP conjugates to bind to CEA was assessed using CEA coated ELISA plates and a anti-mouse IgG(AP labelled, 1/1000) second layer. This was then quantified by the hydrolysis of *p*-nitrophenolphosphate(pNPP) to *p*-nitrophenol at 405 nm. Anti-AP activity was measured by adding AP as a second layer and again measuring colour development at 405 nm.

The targeting of LS174T colonic cancer cells: LS174T cells were grown to 95–100% confluence in 96 well plates and 100 μL of SFM containing the bispecific conjugates (0.5–5 μg mL^{-1}), was added to each well. After irradiation and incubation AP was added, and the amount of AP targeted to the cells was quantitated using pNPP.

Photolysis of conjugates: The NPE–anti-AP antibody conjugates (20 μg mL^{-1} in 0.9% saline) were irradiated in quartz cuvettes with UV-A light from a hand held Spectroline EN-16/F UV lamp. In later experiments the conjugates were added to the plates and irradiated in the presence of the cells. This type of lamp produces UV light over the wavelength range of 325–395 nm with a mercury vapour peak at 365 nm.[10] The total UV-A irradiance of this hand held lamp was 5.45 mW cm^{-2} at a working distance of 0.5 cm.

Keywords: antibody · caging · cancer · photoactivation · UV-a light

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