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Site-specific modification of anti-angiogenesis peptide HM-3 by polyethylene glycol molecular weight of 20 kDa

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HM-3, an RGD modified endostatin-derived polypeptide, is a potent angiogenesis inhibitor synthesized in our laboratory. Its robust inhibitory effects on endothelial cell migration and tumour growth have been demonstrated by *in vivo* and *in vitro* activity assays. However, the drug has relatively short half-life *in vivo*. For the purpose of prolonging HM-3 half-life and retaining the safety and efficacy of the peptide, the study chose methoxy-polyethylene glycol-Succinimidyl Carbonate (SC-mPEG, molecular weight 20 kDa, named SC–mPEG$_{20k}$) to specifically modify its N terminus. Compared with HM-3, the site-specific mono-PEGylated peptide PEG$_{20k}$-HM-3 was shown the same activity in the inhibition of B16F10 tumour *in vivo* (the inhibitory effect of PEG$_{20k}$-HM-3, HM-3 and Taxol were 44.35, 39.68%, respectively), while the frequency of drug-administering reduced from twice a day to once every 3 days. Its rate of *in vitro* degradation in serum was markedly reduced (72.78% could still be detected after 132 h). Histochemistry and immunohistochemistry analysis showed that both HM-3 and PEG$_{20k}$-HM-3 induced large areas of continuous necrosis within tumours and significantly reduced the vessel density compared to control. It might be a breakthrough in PEG modification field to modify a small peptide with a large PEG and reach a good result.

Keywords: angiogenesis inhibition/HM-3/PEG$_{20k}$-HM-3/peptide/SC-mPEG$_{20k}$.

Abbreviations: RGD, Arg-Gly-Asp peptide; SC-mPEG, methoxy-polyethylene glycol-Succinimidyl Carbonate; RP-HPLC, reversed phase-high performance liquid chromatography; Taxol, Paclitaxel.

Currently, angiogenesis has been widely recognized as an important factor controlling the growth of any solid tumour. Since endothelial cells that make up tumour vasculature are genetically stable, cancer treatment targeting at tumour endothelial cells exhibits less toxicity and rarely develops drug resistance compared with traditional chemotherapies. Great interest has been taken in targeting the tumour vasculature and many efforts have been directed towards the development of anti-angiogenic agents. One of the important endogenous angiogenesis inhibitors is endostatin, a protein with a molecular weight of 20 kDa, which was isolated in the culture medium of rat vascular endothelial tumour cells by O'Reilly in 1997 (*1*). Endostatin is the hydrolytic fragment of collagen XVIII. It can prevent blood vessel formation through specifically inhibiting endothelial cell proliferation and migration. Currently, Endostatin is mainly produced by genetic engineering technology. For example, Xu *et al.* (*2*) isolated soluble recombinant human endostatin with anti-angiogenic effect expressed in *Escherichia coli* with molecular chaperone. Dhanabal (*3*) expressed human ES in *Pichia pastoris*. However, since endostatin contains multiple disulfide bonds, the renaturation process is difficult and complicated during manufacture. Thus, the expression product is not stable and the activity is affected after *in vitro* refolding process. Furthermore, the potential targets of endostatin are not clear now. As such, the application of ES is limited (*4*).

In view of this, several groups have focused research on the active fragments of endostatin in order to identify smaller active units. Studies have shown that polypeptides corresponding to the partial sequence of endostatin exhibited higher biological activity (*5*). Wickström *et al.* (*6*) found that the amino acids 50–60 of ES (ES-2) had higher anti-angiogenic activity than the full-length endostatin. However, the *in vivo* activities were not investigated. Xu *et al.* (*7*) established that the *in vivo* activity of ES-2 was not high. But after the introduction of the integrin-binding RGD into ES-2, the modified ES-2 (named HM-3) showed significant anti-tumour results *in vivo*. Safety experiments were conducted to study the possible toxic effects of HM-3, and no any evidence of toxicity was found in the treated animals. These results show that HM-3 is an effective and safe peptide as an anti-cancer molecule in animal model.

However, in the *in vivo* pharmacokinetic studies, HM-3 has relatively short half-life, thus requiring administration twice a day via tail intravenous injection to achieve its optimal *in vivo* anti-tumour efficacy.

Protein modification is an effective way to prolong its half-life. PEGylation is an attractive strategy to enhance the therapeutic efficacy of proteins with a short serum half-life. It can be used to reduce the immunogenicity of proteins, to increase circulation time in the bloodstream, and to lengthen

administration intervals (*8–10*). Recently, several polyethylene glycol (PEG)-conjugated therapeutic proteins have been shown to exhibit clinical properties superior to those of their corresponding unmodified parent proteins (*11*, *12*).

In our previous study (*13*), we had successfully modified HM-3 with methoxy-polyethylene glycolPEG-aldehyde (mPEG-ALD_{10k}). The modified peptide (mPEG-ALD_{10k}-HM-3) can dramatically inhibit angiogenesis on the chorioallantoic membrane of chick embryo (CAM) *in vivo* and its *in vivo* half-life was prolonged by 5.86-fold (162.08 ± 36.57 min) relative to unmodified HM-3. The frequency of drug administering reduced to once daily after modified by mPEG-ALD_{10k}. For the purpose of achieving further diminishing the frequency of drug administering and retaining the safety and efficacy of the peptide, in this study, we designed to modify HM-3 with methoxy-polyethylene glycol-Succinimidyl Carbonate (molecular weight 20 kDa).

Materials and Methods

Materials

HM-3 (99% purity) was chemically synthesized by GL Biochem (Shanghai) Ltd. mPEG$_{20k}$-SC (99% purity) was purchased from Beijing Kaizheng Biotech Development Co. Ltd, batch number 090206. Trifluoroacetate (TFA) and acetonitrile (ACN) were purchased from TEDIA and ROE Scientific Inc., respectively. The male SD rats were purchased from Qinglongshan Laboratory Animal Factory. C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. Mouse anti-HM-3 monoclonal antibody was prepared in our laboratory (Xu, H.-M., unpublished results) and goat anti-CD31 polyclonal antibody was purchased from SANTA CRUZ biotechnology. Horseradish peroxidase-labelled goat anti-mouse secondary antibody was purchased from Boster Biological Company of China.

Methods

PEG modification of HM-3 and purification. A series of reactions were set up using varying stoichiometric ratios ($n_{SC\text{-}mPEG20k}$:$n_{HM\text{-}3}$) of SC -mPEG$_{20k}$ and HM-3 (1 mg, 1 mg/ml) in phosphate buffers (0.1 mol/ml) at pH 5, 6, 7 and 8 and acetate buffers (0.1 mol/ml) at pH 4, 5, 6 and 7.

Sample aliquots were taken after 2, 3 and 5 h, then glycine was added to terminate the reactions. The reaction mixtures were first analysed the modification rate by RP-–HPLC on a C18 column (150 × 4.6 mm, 5 μm, Krosmasil). The mobile phases contained 0.1% TFA in ACN (solution B) and 0.1% TFA in water (solution A), and was applied as a gradient of 10–100% solution B (or 90–10% solution A). Detection wavelength was set at 220 nm. The flow rate was at 1 ml/min, and the running time was 15 min.

The reaction products were further purified by a semi-preparative RP-–HPLC with a C18 column (250 × 10 mm, 5 μm, KYATECH). The mobile phases were 0.1% TFA in ACN (solution B) and 0.1% TFA in water (solution A), and was applied as a gradient of 40–60% solution B (or 60–40% solution A). The detection wavelength was set at 220 nm; the flow rate was at 3 ml/min, and the running time was 20 min.

Validation of reaction products

RP–HPLC. The purity of modification products were analysed by RP–HPLC as described earlier in 'Methods' section and their HPLC chromatograms were compared.

SDS–PAGE electrophoresis and western blot analysis. For the SDS–PAGE identification, reaction mixtures were separated on a SDS–PAGE gel comprised of 5% stacking gel and 10% separation gel. The lane of the marker was stained with coomassie brilliant blue and the lane of PEG$_{20k}$-HM-3 was stained with BaI_2.

After SDS–PAGE electrophoresis, PEG$_{20k}$-HM-3 was further identified by western blot analysis with nitrocellulose western blotting membranes. The staining for PEG$_{20k}$-HM-3 was performed on sections using a specific mouse anti-HM-3 monoclonal antibody of high titre and specificity and horseradish peroxidase-labelled goat anti-mouse secondary antibody, visualized with diaminobenzidine (DAB) chromogen.

In vitro stability of HM-3 and PEG$_{20k}$-HM-3 in plasma

In aliquots of 0.9 ml SD rat plasma blood, 0.1 ml of HM-3 (8 mg/ml), PEG$_{20k}$-HM-3 (97.9 mg/ml, calculated by the same mole of HM-3) were added into separate tubes and incubated in a 37°C water bath.

At 0, 3, 6, 10, 15, 20, 25, 30 and 60 min of HM-3 incubation, 0.1 ml aliquots were taken and precipitated with perchloric acid. After centrifugation to obtain the supernatants, RP–HPLC analysis was carried out to measure the drug concentration in the supernatants, and retention percentages were calculated.

For PEG$_{20k}$-HM-3 incubation, the samples were taken at 0, 12, 24, 48, 72, 84, 102, 108, 120 and 132 h. And the treatments of the aliquots were the same as HM-3.

The anti-tumour activity of PEG$_{20k}$-HM-3 in vivo

Tumour implantation. B16F10 mouse melanoma cell line was grown in culture. Cell lines were washed with phosphate buffered saline (PBS), dispersed in a 0.05% trypsin solution, and re-suspended. After centrifugation at 1000 r.p.m. for 5 min, the cell pellets were re-suspended in PBS and adjusted to a concentration of 5×10^6 cells/ml. C57BL/6 female mice (5- to 6-weeks old) were implanted subcutaneously (s.c.) on the mid-right side with 5×10^5 B16F10 cells in 0.1 ml PBS.

C57BL/6 female mice treatment. After the average tumour volume of B16F10 mouse melanoma reached 50–150 mm^3, mice were randomly divided into different groups with eight mice per group. Group 1 received PBS. Group 2 was injected with Paclitaxel (Taxol, provided by Tai ji Pharmaceutical Co. Ltd., Chendu in Sichuan province of China) once daily at a dose of 3 mg/kg in PBS. Group 3 and Group 4 received HM-3 twice a day at a dose of 3 mg/kg/day and PEG$_{20k}$-HM-3 once every 3 days at a dose of 36.7 mg/kg which was the same moles as HM-3, respectively. Group 5 received SC-mPEG$_{20k}$ once every 3 days at a dose of 33.7 mg/kg which was the same moles as PEG$_{20k}$-HM-3. All of the mice were injected subcutaneously distant from the tumour sites.

Measurement of tumour growth. Tumours were measured individually with a vernier caliper. Volumes were determined using the formula: tumour volume = length × width^2 × 0.52. Therapeutic effects on tumour growth were expressed as mean tumour volume versus time, calculated as $(1-T/C) \times 100\%$, where T = treated tumour volume and C = control tumour volume. For example, if the volume of the treated tumour was 40% of that of the control on a given day, tumour suppression in the treated group was regarded as 60%.

Histochemistry and immunohistochemistry

Mice were euthanized 2 weeks post-treatment, and tumour tissue was collected, fixed with 4% formaldehyde, embedded in paraffin and sectioned for haematoxylin and eosin (H&E) staining and immunohistochemical staining for CD31. H&E staining was performed according to standard histological procedures. Necrosis and vascularization in tumour tissue were observed under a light microscope. Vascular structures in tumours were evaluated by immunohistochemical staining of CD31 with goat anti-CD31 polyclonal antibody. Briefly, staining for CD31 was performed on sections using their specific primary antibodies and horseradish peroxidase-labelled rabbit anti-goat secondary antibody, visualized with diaminobenzidine (DAB) chromogen, counterstained with haematoxylin, and observed with a microscope.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was assessed using the Student t-test. For all statistical comparisons, treated groups were compared with PBS-treated controls. $P < 0.05$ was considered statistically significant.

Results

PEG modification of HM-3 and purification of the modification product

A series of modification reactions were set up by mixing SC-mPEG$_{20k}$ and HM-3 at different molar ratios under conditions with varying different buffer, buffer pH and reaction time. The reaction mixtures were analysed by RP–HPLC to assess effects on the reactions by different conditions. The results (Fig. 1A and B) showed that there was only one single peak for the modification product, and the maximal modification rate of 99.08% was achieved when the substrate molar ratio (n $_{SC\text{-}mPEG20k}$:n $_{HM\text{-}3}$) was set at 3, and the reactions were incubated for 3 h at 4°C in a pH 8 phosphate buffer calculated by the change of peak areas. The substrate molar ratio and pH of substrate buffer have the most profound effects on the modification rate. However, the modification rate was also high when the substrate molar ratio (n $_{SC\text{-}mPEG20k\text{-}}$:n $_{HM\text{-}3}$) set at 2 (86.27%).

Considering by-product might be caused by high molar ratio (n $_{SC\text{-}mPEG20k\text{-}}$: n $_{HM\text{-}3}$) (*14*, *15*) and the cost, we chose molar ratio (n $_{SC\text{-}mPEG20k}$: n $_{HM\text{-}3}$) at 2 and the reactions were incubated for 3 h at 4°C in a pH 8 phosphate buffer for modification.

Reaction products were purified through semi-preparative RP–HPLC. Analyses showed that the purity of the products was 96% (Fig. 1C).

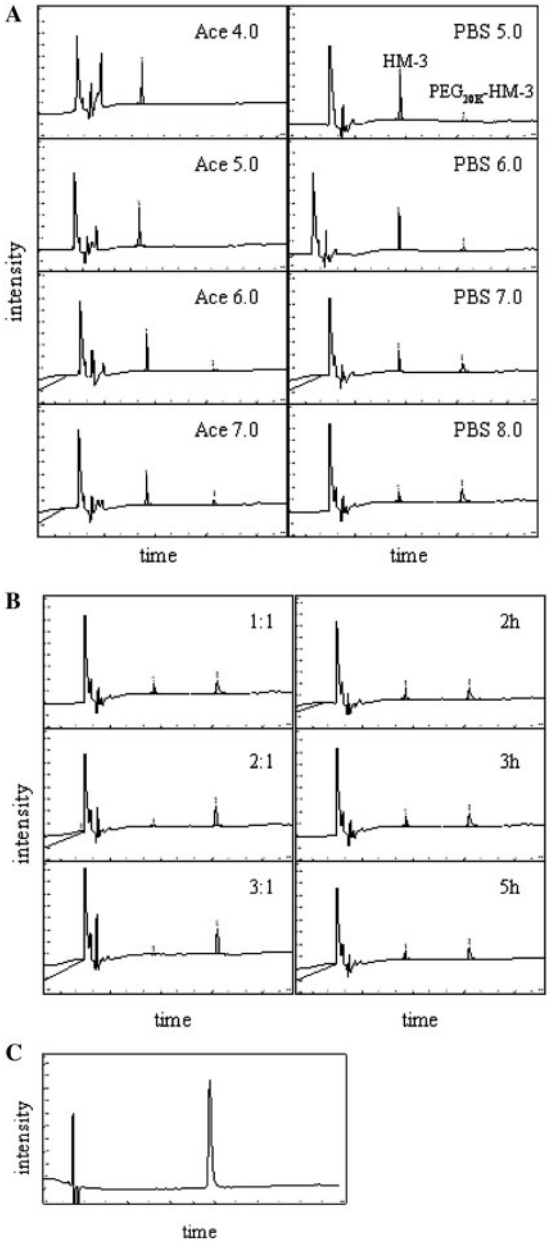

Fig. 1 RP–HPLC analysis of reaction mixtures obtained by reaction of SC-mPEG$_{20k}$ with HM-3 at different buffer pHs, molar ratios (n $_{SC\text{-}mPEG20k}$: n $_{HM\text{-}3}$) and reaction times as indicated. Analysis was performed on C18 column with water/ACN/TFA as eluent and a flow rate of 1 ml/min, and peaks were monitored at 220 nm (A and B). RP–HPLC analysis of the PEGylated HM-3 Purification (C).

Product validation

RP–HPLC chromatograms (Fig. 1C) revealed the presence of a single peak, confirming that only one modification product was generated from the modification reaction.

After HM-3 modification by SC-mPEG$_{20k}$, the product was showed as a single band by Electrophoresis analysis (Fig. 2).

Western blot (Fig. 2) revealed the immunogenicity of the modified peptide did not change.

In vitro *serum stability of PEG$_{20k}$-HM-3*

After the drug was mixed with serum and incubated at 37°C, RP–HPLC analysis was performed to measure drug retention percentage in the serum at different time points. The degradation rate of HM-3 in serum was very high, as HM-3 became undetectable after only 25 min incubation. In contrast, 90.81% of PEG$_{20k}$-HM-3 remained intact after 48 h (Fig. 3), and 72.78% could still be detected after 132 h. These results demonstrate that after modification, the polypeptide became less susceptible to the degradative enzymes in the serum and its stability was markedly enhanced.

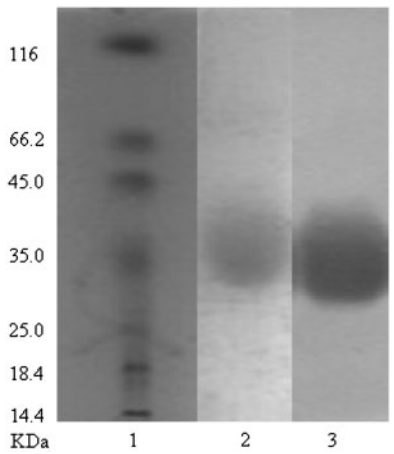

Fig. 2 SDS–PAGE and western blot analysis of the PEGylated HM-3. Lane 1, Molecular weight markers. Lane 2, SDS–PAGE of PEG$_{20k}$-HM-3. Lane 3, western blot of PEG$_{20k}$-HM-3.

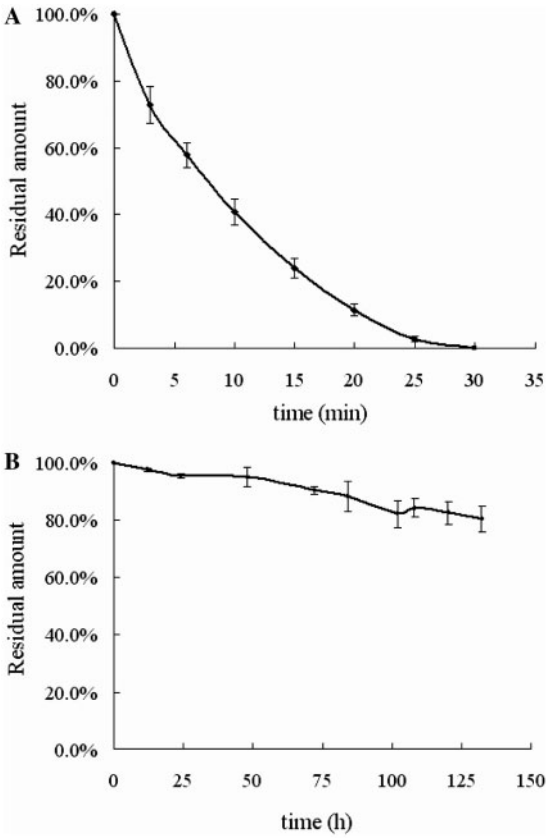

Fig. 3 Residual amounts of equal molar HM-3 and PEG_{20k}-HM-3 in plasma *in vitro* at 37°C. HM-3 became undetectable after only 25 min incubation (A), while 72.78% of PEG20k-HM-3 could still be detected after 132 h incubation (B).

In vivo *anti-tumour activity*

During the treatment of the tumour-bearing mice, the volumes of the tumours were measured every 2 days. After treated for 2 weeks, the whole tumours were teared down and weighed.

In *in vivo* study of comparing the activity of HM-3 and PEG_{20k}-HM-3. The size of implanted mouse melanoma tumours in the groups treated with HM-3 and PEG_{20k}-HM-3 were significantly smaller than that in the PBS control group, and the inhibitory effect of PEG_{20k}-HM-3, HM-3 and Taxol were 44.35, 39.68 and 31.33, respectively. PEG_{20k}-HM-3-treated group had no significant difference from HM-3-treated group in the tumour size, and even at the same moles, the tumour size of PEG_{20k}-HM-3-treated group was smaller than HM-3-treated group (Fig. 4). Besides, the SC-$mPEG_{20k}$ group showed no difference from the PBS control group, which exhibited no anti-tumour efficiency of SC-$mPEG_{20k}$.

Histochemistry and immunohistochemistry

We examined the effect of the peptides on tumour tissue. HM-3 and PEG_{20k}-HM-3 displayed more severe tissue necrosis than the control (Fig. 5A–C). The control displayed necrosis interspersed with viable tumour cells, whereas HM-3 and PEG_{20k}-HM-3 induced large areas of continuous necrosis within tumours. We also examined the effect of Taxol (Fig. 5D), and found that although it induced

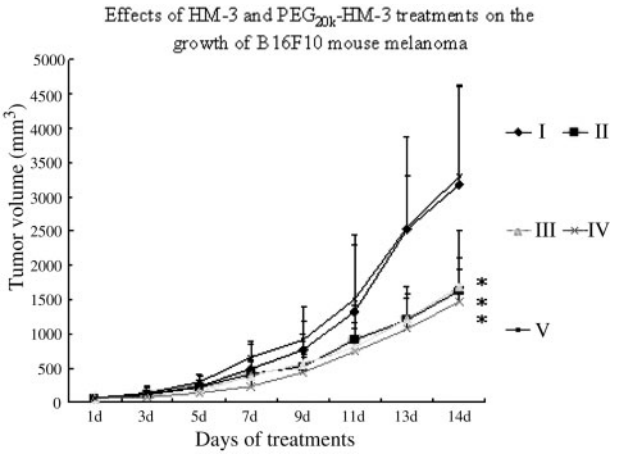

Fig. 4 Therapeutic effects of peptides on the growth of tumour. (I) represents control, once a day. (II) displays the positive drug taxol, once a day. (III) displays HM-3 at the dose of 3 mg/kg twice a day. (IV) displays PEG_{20k}-HM-3 at the dose of 36.7 mg/kg once every 3 days. (V) displays SC-$mPEG_{20k}$ at the dose of 33.7 mg/kg once every 3 days. Each point represents mean ± SD of each group (*$P<0.05$ vs control).

large areas of continuous necrosis within tumours but had no effect on blood vessels. We then counted the number of visible blood vessels in CD31 staining, and found that both HM-3 and PEG_{20k}-HM-3 significantly reduced the vessel density compared to control (Fig. 5E, F and G) ($P<0.01$). The angiogenesis inhibition activity of PEG_{20k}-HM-3 was not changed or degraded compared with HM-3. The blood vessels were as follows: control 14.6923 ± 4.0573, HM-3 5.7803 ± 2.0309 and PEG_{20k}-HM-3, 4.8276 ± 1.7540.

Discussion

HM-3, a peptide synthesized in our laboratory, exhibits significant anti-tumour activity in animal models. However, *in vivo* pharmacokinetic study showed that similar to other proteins and peptides, the half-life of HM-3 was relatively short (10–27 min). Thus, HM-3 needs to be given twice a day for pharmacodynamic studies in animal models. In order to extend half-life and reduce the administration frequency without affecting its activity, we have performed PEG modification since 2006 in our group. Two types of PEG molecules have been tested including mPEG-ALD and SC-mPEG, with molecular weight of 2, 5, 10 and 20 kDa. Reaction conditions (including buffer type, pH, molar ratio, time, temperature, etc.) as well as separation and purification conditions have been investigated regarding their effects on *in vivo* activity and *in vitro* half-life of HM-3. Up to now, SC-$mPEG_{20K}$-HM-3 has been proved to be the most ideal modification product. After modification, the regimen of HM-3 was changed from twice a day to once every 3 days. Preliminary *in vitro* plasma half-life determination also indicated that its half-life was extensively extended. Based on the last 3 years of research, we have also accumulated some experience. Here, we would like to share what we have learned from our studies with other researchers.

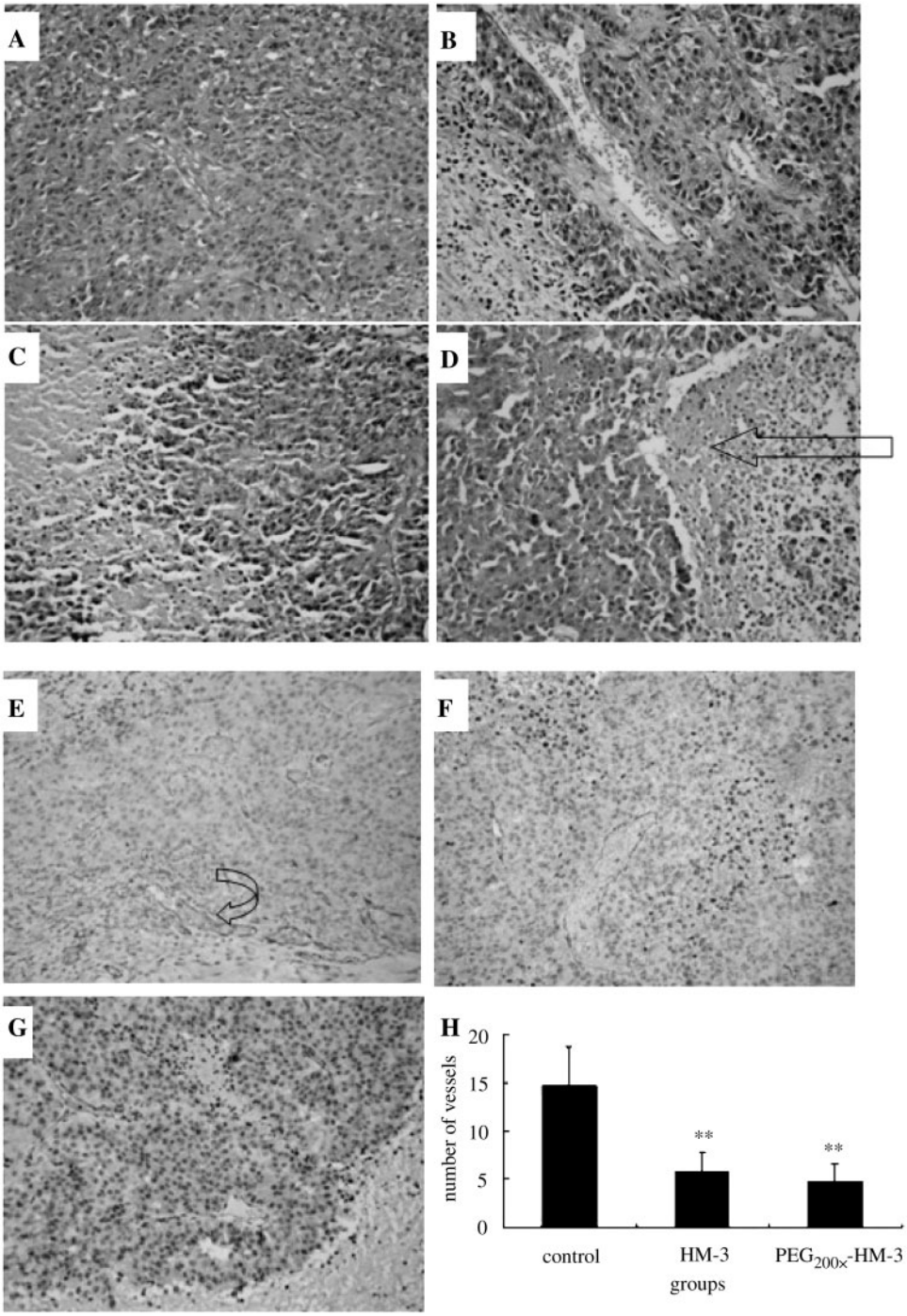

Fig. 5 Necrosis areas within tumour and tumour blood vessel density. The figure (A, B, C and D) shown by H&E staining observed with high microscope (200 × original magnifications). PBS (A) treatment exibits incomplete tissue necrosis interspersed with viable tumour cells. (B) represents taxol treatment, (C) epresents HM-3 treatment, (D) represents PEG_{20k}-HM-3 treatment, they all showing continuously extensive necrosis area. (E–G) Tumour blood vessel density. (E) represents PBS, (F) represents HM-3, and (G) PEG_{20k}-HM-3 treatment. (H) represents number of vessels of control, HM-3 and PEG_{20k}-HM-3 treatment. Blood vessels in CD31 staining were indicated with the circular arrow and tumour necrosis areas were indicated with the straight arrow (200 × original magnifications). Results were expressed as mean (SD) of nine regions of three sections per group ($*P<0.05$, $**P<0.01$ versus control).

First, the product obtained from mPEG-ALD modification exhibited activity and extended half-life. However, toxicity was also observed during *in vivo* pharmacodynamics study, resulting in high mortality. Such toxicity might be caused by the residue of a small amount of reducing agent sodium cyanoborohydride.

Second, modification with small-molecule PEG, such as 2 kDa PEG, had no significant effect on the half-life of the peptide. The major reason might be that after modification with small molecule PEG, the metabolism of HM-3 was still very fast. Thus no significant effect was observed regarding the extension of HM-3 half-life.

Third, generally speaking, modification with large-molecule PEG may result in reduced activity. The activity of modification products of anti-tumour proteins and peptides is closely associated with organism resistance, PEG mass and shape, stability of PEG-protein or PEG-peptide bond, and changes in the spatial structure of modified products. In our study, the activity of PEG_{20K}-HM-3 did not change. This might be due to the fact that the loss of biological activity was compensated for by the prolonged body-residence time, as a result of the increased stability and higher hydrodynamic volume after modification. In addition, HM-3 (highly soluble in water) and PEG are hydrophilic molecules. Thus, in water system, HM-3 was not surrounded by chain-like PEG and the activity sites were not masked. Rather, HM-3 remained its chain structure and its activity was not decreased, but the half-life was largely extended.

Fourth, in this study, the theoretical molecular weight of the modification product was 21,780 Da. However, molecular weight as shown in electrophoresis and western blot analysis was not consistent with theoretical one. This was due to the larger hydraulic radius of PEG molecule coupling to HM-3, resulting in reduced electrophoretic migration speed. Thus, effective separation could not be obtained and measured molecular weight was not consistent with actual one.

Fifth, PEG is obtained by chemical synthesis and is polydisperse, which means that polymer's batch is composed of molecules having different number of monomers, yielding a Gaussian distribution of the molecular weights. The modification target in this study was *N*-amino and the peptide HM-3 do not have lysine (the modification targets for mPEG-ALD and SC-mPEG are *N*-amino and lysine, respectively). Thus, the modification was specific. However, analysis of PEG_{20K}-HM-3 by MALDI-TOF indicated existence of impurity peaks. There might be two reasons: one was the quality of PEG. For example, low uniformity, wide distribution of molecular weight, or existence of PEG dimmers; the other reason might be that small amount of modification products existed as dimmers.

Lastly, during the pharmacodynamic study in animal models, we found that it was appropriate to treat animal models transplanted with B16F10 cells at early stage. However, if those animals were treated after tumour volume reached 100–300 mm^3 according to the methods reported by others, broken tumour and bleeding were always observed and animals died (including positive control group and saline group). As a result, the therapeutic effects could not be followed very well. Thus, we suggested that the drug should be administered 2–3 days after transplantation B16F10 cells or when tumour volume is $<100\ mm^3$.

In summary, PEG modification is a successful method to change the half-life of drugs and reduce the immunogenicity. Many PEG modified proteins and peptides have successfully come into the market. Our study might be a breakthrough in PEG modification field to modify a small peptide with a large PEG and reach a good result.

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