

跨膜型超抗原金黄色葡萄球菌肠毒素 A 融合蛋白的抗肿瘤作用

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摘要: 目的 为扩大超抗原金黄色葡萄球菌肠毒素 A(SEA)的抗瘤谱,制备跨膜型 SEA 融合蛋白,研究该蛋白制备的肿瘤疫苗的抗肿瘤作用。方法 在荷 B16 黑色素瘤的 C57BL/6 小鼠上,观察跨膜型 SEA 融合蛋白制备的肿瘤疫苗对荷瘤小鼠的免疫治疗作用和免疫保护作用,并通过乳酸脱氢酶(LDH)释放法检测治疗组和免疫组小鼠脾细胞的天然杀伤细胞(NK)和细胞毒性 T 细胞(CTL)活性。结果 融合蛋白制备的肿瘤疫苗能够显著抑制荷瘤小鼠肿瘤的生长,并延长其生存期,其脾细胞的 NK 和 CTL 活性显著增强。同时,该肿瘤疫苗对同种肿瘤细胞攻击可产生较强的免疫保护作用。结论 跨膜型 SEA 融合蛋白制备的肿瘤疫苗具有显著的抗肿瘤作用,可有效激发荷瘤小鼠机体的特异性和非特异性抗肿瘤免疫应答,增强 CTL 和 NK 活性。

关键词: 超抗原; 跨膜; 融合蛋白; 免疫疗法; T 淋巴细胞

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1989 年,White 等^[1]提出了超抗原(superantigen, SA_g)的概念,超抗原无需抗原提呈细胞(antigen presenting cells, APC)加工而以完整的蛋白形式与主要组织相容性抗原(major histocompatibility complex, MHC) II 类分子结合,激活所有携带特殊 T 细胞受体

(T-cell receptor, TCR) V β 片段的 T 细胞,激活 T 细胞的克隆数约是普通抗原的 1000 倍,对靶细胞产生强烈的杀伤作用,称之为 SA_g 依赖的细胞介导的细胞毒作用(superantigen-dependent cell-mediated cytotoxicity, SDCC)^[1,2]。超抗原强烈激活 T 细胞的特性启发人们将其用于抗肿瘤治疗,但超抗原并不能直接用于抗肿瘤,大多数肿瘤细胞并不表达 MHC II 类抗原,而表达 MHC II 类分子的正常细胞如 APC 也能成为超抗原的靶细胞而受到攻击。为了使超抗原发挥特异性的抗肿瘤作用,Dohlsten 等^[3]制备了人结肠癌反应性单克隆抗体 C215 的 Fab 段与金黄色葡萄球菌肠毒素 A(staphylococcal enterotoxin A, SEA)的融合蛋白(C215Fab-SEA),C215Fab-SEA 可特异性杀伤表达 C215 抗原的肿瘤,但单克隆抗体导向超抗原的抗肿瘤谱很窄,一种单克隆抗体只能靶向一种特异的肿瘤组织,缺乏抗瘤广谱性。也有学者用交联剂使 SEB 结合在 Meth A 肿瘤细胞表面^[4],能有效刺激 V β 8⁺ T 细胞的增殖,肿瘤生长明显受到抑制,但化学偶联的稳定性欠佳。针对目前超抗原用于抗肿瘤治疗的现状,本文将超抗原 SEA 与跨膜区(transmembrane, TM)编码序列融合,构建了跨膜型超抗原 TM-SEA 的融合蛋白并制备了肿瘤疫苗,扩大了其抗肿瘤作用的范围,并以小鼠黑色素瘤为肿瘤模型,研究其抗肿瘤作用并初步探讨了作用机制。

1 材料与方 法

1.1 材料

跨膜型超抗原融合蛋白由本实验室制备并纯化^[5]。丝裂霉素 C(mitomycin C, MMC)购自 Fluka 公司,乳酸脱氢酶(lactate dehydrogenase, LDH)释放测定试剂盒购自 Promega 公司。小鼠黑色素瘤细胞系 B16,来源于 C57BL/6 小鼠。YAC-1 细胞系,鼠 NK-敏感性淋巴瘤细胞。EL4 小鼠 T 淋巴瘤细胞系,来源于 C57BL/6 小鼠。6~8 周龄 C57BL/6 小鼠(H-2^b),共 80 只,购于中国科学院上海实验动物中心。

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1.2 小鼠脾细胞的分离和效应细胞的制备

脱颈椎处死 C57BL/6 小鼠, 75% 乙醇浸泡消毒, 无菌条件下取出脾脏, 用磷酸缓冲盐溶液 (PBS) 冲洗 1 次, 然后将其研碎, 并悬浮于无血清的 RPMI 1640 培养基中, 静置 10 min 使大块组织沉降后, 将上清过 120 目筛网, $500 \times g$ 离心 10 min, 弃上清, 加入 Tris-NH₄Cl 缓冲液, 混匀后室温放置 5 min, 加入等量的无血清培养基, 离心弃上清, 再用无血清的 RPMI 1640 培养基洗涤 3 次, 然后用 RPMI 1640 完全培养基配成 $1 \times 10^{10} \text{ L}^{-1}$ 的细胞悬液待用。

1.3 脾细胞天然杀伤细胞活性的检测

取新传代 12~24 h 的 YAC-1 细胞, 用 RPMI 1640 完全培养基将细胞浓度调整至 $1 \times 10^8 \text{ L}^{-1}$, 作为靶细胞, 接种于 96 孔中, 每孔 0.1 mL, 按效靶比 100:1, 50:1, 25:1 的比例加入脾细胞, 用 LDH 释放法测定天然杀伤细胞 (natural killer, NK) 活性^[6]。

1.4 细胞毒性 T 细胞的诱导与杀伤活性的测定

将分离的小鼠脾淋巴细胞, 与 $100 \text{ mg} \cdot \text{L}^{-1}$ MMC 灭活的 B16 细胞置于 37°C , 5% CO₂ 细胞培养箱中共同孵育 7 d, 加入重组的小鼠 IL-2 ($20 \text{ kU} \cdot \text{L}^{-1}$), 收集后作为 CTL 效应细胞, B16 细胞作为杀伤的靶细胞 (浓度调整至 $1 \times 10^8 \text{ L}^{-1}$, 每孔 0.1 mL), 按效靶比 50:1, 25:1, 12.5:1 的比例接种于 96 孔板, 用 LDH 释放法测定细胞毒性 T 细胞 (cytotoxicity T lymphocyte, CTL) 活性^[6]。

1.5 B16 肿瘤疫苗的制备

B16 细胞与终浓度为 $100 \text{ mg} \cdot \text{L}^{-1}$ MMC 于 37°C 共同孵育 1 h, PBS 洗涤 3 次后, 加入适量体积的 PBS 使细胞的浓度为 $1 \times 10^{10} \text{ L}^{-1}$ 。使用时于瘤内注射 0.1 mL, 即 1×10^6 细胞。

1.6 B16-SEA 及 B16-TM-SEA 肿瘤疫苗的制备

B16 细胞与终浓度为 $100 \text{ mg} \cdot \text{L}^{-1}$ MMC 于 37°C 共同孵育 1 h, PBS 洗涤 3 次后, 与终浓度为 $45 \text{ nmol} \cdot \text{L}^{-1}$ 的单纯 SEA 蛋白或 TM-SEA 融合蛋白共同孵育 4 h, 制备 B16-SEA 及 B16-TM-SEA 肿瘤疫苗, PBS 洗涤 3 次, 加入适量体积的 PBS, 使细胞的浓度为 $1 \times 10^{10} \text{ L}^{-1}$ 。使用时于瘤内注射 0.1 mL, 即 1×10^6 细胞。

1.7 肿瘤疫苗对荷瘤小鼠的免疫治疗

于 C57BL/6 小鼠的右后肢 sc 1×10^5 个野生型 B16 细胞, 体积为 0.1 mL。荷瘤 3 d 后将小鼠随机分成 PBS (空白对照)、B16 疫苗、B16-SEA 疫苗、B16-TM-SEA 疫苗 4 组, 注射量每组均为 0.1 mL, 每组 8

只, 3 d 注射 1 次瘤苗, 共 4 次。

每 2 d 用游标卡尺测量并记录各组荷瘤小鼠瘤体的大小, 治疗结束后 5 d, 每组取 3 只小鼠, 处死后取脾脏制备淋巴细胞, 检测 NK 和 CTL 活性。对剩余的 5 只小鼠, 观察其生存期。

1.8 肿瘤疫苗对肿瘤细胞攻击的免疫保护作用

将 C57BL/6 小鼠随机分成 PBS (B16 细胞攻击)、B16 疫苗 (B16 细胞攻击)、B16-SEA 疫苗 (B16 细胞攻击)、B16-TM-SEA 疫苗 (B16 细胞攻击)、PBS (EL-4 细胞攻击)、B16-TM-SEA 疫苗 (EL4 细胞攻击) 6 组, 每组 8 只, 于其右腹股沟皮下分别注射不同的疫苗 (剂量同前), 每周 1 次, 共 3 次。

免疫结束后 d 7, 前 4 组小鼠均用野生型 B16 细胞攻击; 第 5, 6 组小鼠用 EL4 细胞攻击。攻击时 B16 细胞数量为每鼠 1×10^5 , EL-5 细胞数量为每鼠 5×10^5 。肿瘤细胞攻击后 1 周, 开始观察并记录成瘤结果, 游标卡尺测量各组小鼠形成肿瘤的大小。免疫结束后 5 d, 每组取 3 只小鼠, 处死后取脾脏淋巴细胞, 检测 CTL 活性。对剩余的 5 只小鼠, 观察其生存期。

1.9 统计学处理

所有相关数据均采用 SPSS 软件包进行统计学分析, 生存率比较采用 Log-Rank 检验, 其他计量资料采用方差分析 (F 检验) 和两两比较 q 检验。

2 结果

2.1 肿瘤疫苗对荷瘤小鼠肿瘤生长的抑制作用

于 C57BL/6 小鼠的右后肢 sc 1×10^5 个野生型 B16 细胞。5~7 d 后, 发现有可触及的肿瘤。24 d 时, B16-TM-SEA 疫苗治疗组小鼠的平均肿瘤直径显著小于 PBS 组、B16 和 B16-SEA 疫苗组 ($P < 0.01$, 图 1); B16 和 B16-SEA 疫苗组小鼠的平均肿瘤大小与 PBS 组比较无显著性差异 (图 1)。

2.2 荷瘤小鼠脾细胞的天然杀伤细胞活性

图 2 结果表明, 与 PBS 对照组相比, B16-TM-SEA 疫苗治疗组小鼠的 NK 活性显著性增高 ($P < 0.05$), 而 B16 和 B16-SEA 疫苗组小鼠的 NK 活性无显著性改变。

2.3 荷瘤组小鼠脾细胞的细胞毒性 T 淋巴细胞活性

图 3 结果表明, 与 PBS 组、B16 及 B16-SEA 肿瘤疫苗组比较, B16-TM-SEA 肿瘤疫苗组小鼠脾细胞的 CTL 活性显著升高 ($P < 0.01$, 图 3); 而且当 E:T =

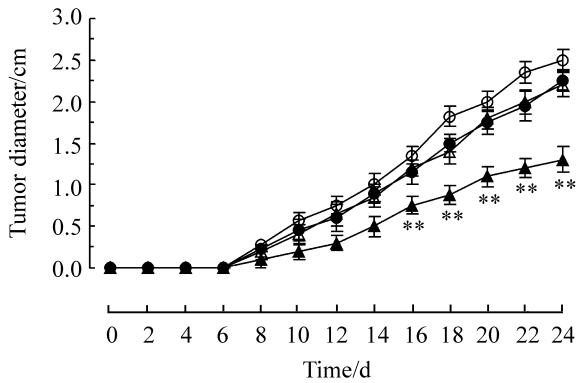


Fig 1. Curves of tumor growth after inoculation of melanoma B16 in mice treated with different agents. C57BL/6 mice were inoculated subcutaneously with 1×10^5 B16-C215 cells on day 0. The tumor-bearing C57BL/6 mice were divided into 4 groups ($n = 8$) and injected intratumorally with PBS 0.1 mL(○), B16 vaccine 0.1 mL(●), B16-SEA vaccine 0.1 mL(△), B16-TM-SEA vaccine 0.1 mL(▲), respectively. Injection of the vaccine was performed 4 times every 3 d. The length and width of the tumor mass were measured with caliper every other day. SEA: staphylococcal enterotoxin A. TM: transmembrane. $\bar{x} \pm s$, $n = 8$. ** $P < 0.01$, compared with PBS group.

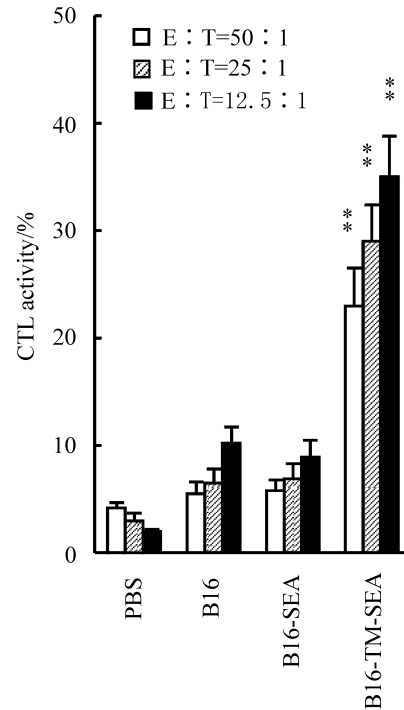


Fig 3. Induction of cytotoxicity T lymphocyte (CTL) activity of the B16-bearing mice treated with different agents. Lymphocytes isolated from tumor-bearing mice after various therapies were cocultured with inactivated B16 ($MMC 100 \text{ mg} \cdot \text{L}^{-1}$) for 7 d in the presence of recombinant murine IL-2 ($20 \text{ kU} \cdot \text{L}^{-1}$) and then collected as CTL effector cells. The B16 melanoma cells were used as target cells. The CTL activity was determined at E:T ratio of 12.5:1, 25:1, 50:1 by LDH release assay. $\bar{x} \pm s$, $n = 3$. ** $P < 0.01$, compared with PBS group.

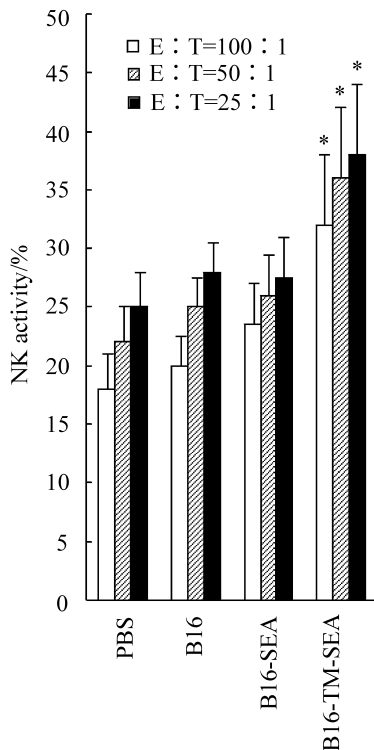


Fig 2. Natural killer (NK) activity of splenocytes derived from the B16-bearing mice treated with different agents. Splenic lymphocytes, which were isolated from sacrificed tumor-bearing mice of different groups 5 d after the last injection, were used in cytolytic assays against YAC-1 cells at E:T ratios of 25:1, 50:1, 100:1 by LDH release assay. $\bar{x} \pm s$, $n = 3$. * $P < 0.05$, compared with PBS group.

50:1时,CTL活性升高最为显著。而 B16 肿瘤疫苗组和 B16-SEA 肿瘤疫苗组小鼠脾细胞的 CTL 活性与较 PBS 组略高,但差异无显著性($P > 0.05$)。

2.4 荷瘤小鼠的生存期

对每组剩余的 5 只小鼠观察其生存期的结果表明,B16-TM-SEA 疫苗治疗组小鼠较 B16 组、B16-SEA 疫苗治疗组和 PBS 组小鼠的生存期显著性延长($P < 0.01$,图 4)。荷瘤 35 d 后,B16-TM-SEA 疫苗组小鼠的生存率为 80%,而其他组小鼠的生存率为 0。

2.5 各免疫组小鼠对肿瘤细胞攻击后的肿瘤生长曲线

用野生型 B16 细胞攻击的 B16-TM-SEA 疫苗免疫组小鼠与 B16 组、B16-SEA 疫苗免疫组、PBS 组小鼠,以及与 EL4 细胞攻击的 B16-TM-SEA 疫苗免疫组比较,小鼠肿瘤显著性缩小($P < 0.01$,图 5)。与

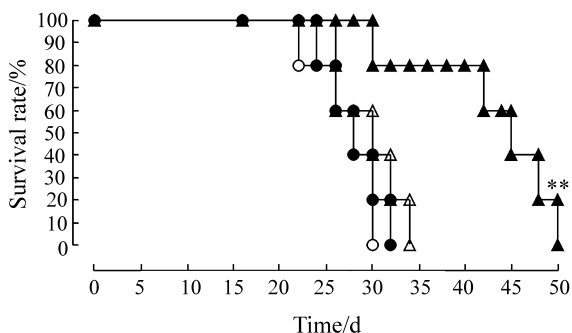


Fig 4. Survival time of B16-bearing mice treated with different agents. The treatments and the symbols were the same as described in Fig 1. Tumor-bearing mice in each group were observed for their survival time. $\bar{x} \pm s$, $n = 5$. ** $P < 0.01$, compared with PBS group.

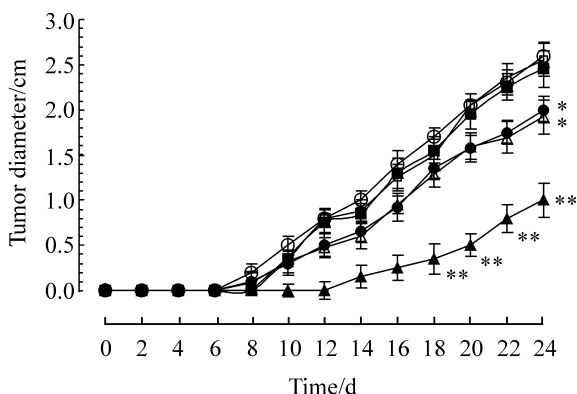


Fig 5. Curves of tumor growth of the immunized mice challenged with tumor cells. C57BL/6 mice were divided into 6 groups ($n = 8$): unvaccinated (PBS) challenged with 1×10^5 B16 cells (\circ); immunized with B16 vaccine challenged with 1×10^5 B16 (\bullet); immunized with B16-SEA vaccine challenged with 1×10^5 B16 (\triangle); immunized with B16-TM-SEA vaccine challenged with 1×10^5 B16 (\blacktriangle); unvaccinated (PBS) challenged with 5×10^5 EL4 lymphoma cells (\square); immunized with B16-TM-SEA vaccine challenged with 5×10^5 EL4 lymphoma cells (\blacksquare). The vaccination was performed at weekly intervals for three consecutive weeks. Tumor challenge was initiated by injection of tumor cells subcutaneously into the rear leg of the mice in different groups 1 week after the last vaccination. The length and width of the tumor mass were measured caliber every other day after tumor challenge. $\bar{x} \pm s$, $n = 8$. * $P < 0.05$, ** $P < 0.01$, compared with PBS group.

PBS组和用EL4攻击的B6-TM-SEA疫苗免疫组小鼠相比,B16疫苗免疫组和B16-SEA疫苗免疫组的肿瘤存在不同程度的缩小($P < 0.05$)。结果表明,使

用B16-TM-SEA肿瘤疫苗免疫的C57BL/6小鼠,可以免受同种肿瘤细胞的攻击,获得较强的特异性免疫保护作用。

2.6 免疫小鼠脾细胞的细胞毒性T淋巴细胞活性

免疫结束后5d,分别选取PBS组、B16疫苗组、B16-SEA疫苗组和B16-TM-SEA疫苗组的小鼠各3只,常规制备其脾细胞,LDH释放法测定CTL的活性。与其他3组比较,B16-TM-SEA疫苗免疫组小鼠脾细胞的CTL活性显著升高($P < 0.01$,图6);而且当E:T=50:1时,CTL活性升高最为显著。B16肿瘤疫苗组和B16-SEA疫苗组小鼠脾细胞的CTL活性与PBS组比较,也有明显升高($P < 0.05$,图6)。

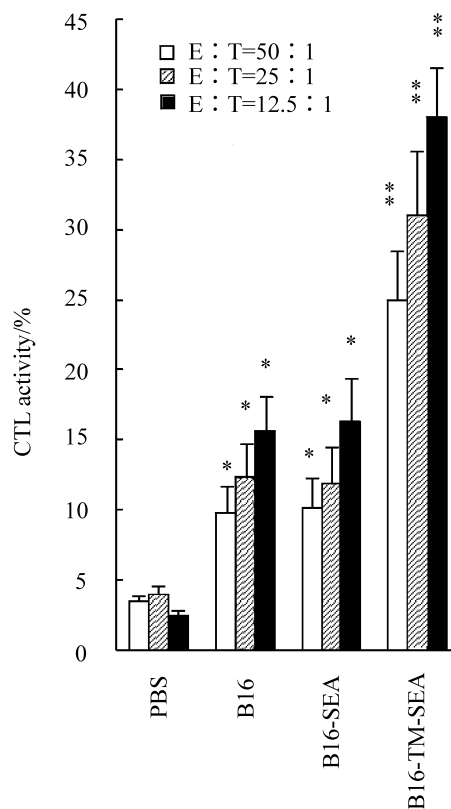


Fig 6. Induction of CTL activity of the mice immunized with different agents. Lymphocytes isolated from C57BL/6 mice 5 d after the last vaccination from different groups were cocultured with inactivated B16 ($100 \text{ mg} \cdot \text{L}^{-1}$) for 7 d in the presence of recombinant murine IL-2 ($20 \text{ kU} \cdot \text{L}^{-1}$) and then collected as CTL effector cells. The B16 melanoma cells were used as target cells. The CTL activity was determined at E:T ratio of 12.5:1, 25:1, 50:1 by LDH release assay. $\bar{x} \pm s$, $n = 3$. * $P < 0.05$, ** $P < 0.01$, compared with PBS group.

2.7 免疫组小鼠的生存期

每组剩余的5只小鼠,用于观察其生存期。结

果表明:用 B16-TM-SEA 疫苗免疫的小鼠,当用野生型 B16 细胞攻击时,此组小鼠的生存期较 PBS 组和用 EL4 肿瘤细胞攻击组的小鼠的生存期显著性延长($P < 0.01$,图 7),其中有 40%的小鼠无瘤生存。

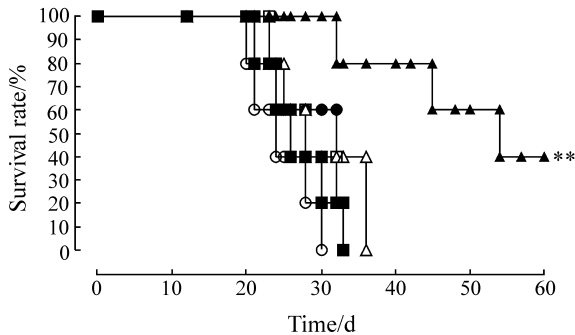


Fig 7. Survival time of the immunized mice challenged with tumor cells. The treatments and the symbols were the same as described in Fig 5. Tumor-bearing mice in each group were observed for their survival time. $\bar{x} \pm s$, $n = 5$. ** $P < 0.01$, compared with PBS group.

3 讨论

本研究针对超抗原的研究现状及存在的问题,为了拓展 SA_g 的抗肿瘤谱,将超抗原 SEA 与 TM 编码序列融合,制备跨膜型超抗原 TM-SEA 的融合蛋白及其肿瘤疫苗,并对其抗肿瘤作用进行了研究。本研究中生产的融合蛋白具有锚定在肿瘤细胞膜上的能力,在制备肿瘤疫苗时,采用了简便的共同孵育的方法,使超抗原 SEA 锚定在肿瘤细胞膜上,基因转染比较费时费力^[7],而锚定的方法仅用 4 h 即可制备成所需要的肿瘤疫苗。

作者应用此肿瘤疫苗研究其对荷黑素瘤 C57BL/6 小鼠的治疗作用及其免疫保护作用,并观察了治疗后小鼠 NK 细胞和 CTL 活性的改变,以探讨其作用机制。结果发现,治疗组小鼠荷瘤 24 d 时,B16-TM-SEA 肿瘤疫苗治疗组小鼠的平均肿瘤直径显著性小于 PBS 组、B16 和 B16-SEA 肿瘤疫苗组。同时,与 PBS 对照组、B16 疫苗、B16-SEA 疫苗组相比,B16-TM-SEA 肿瘤疫苗组小鼠脾细胞的 NK 活性显著性增高,脾细胞的 CTL 活性也有一个显著性的升高,当 E:T = 50:1 时,CTL 活性升高最为显著。B16-TM-SEA 肿瘤疫苗治疗组小鼠较 B16 组、B16-SEA 肿瘤疫苗治疗组小鼠和 PBS 对照组小鼠的生存期显著性延长,表明 B16-TM-SEA 肿瘤疫苗对小鼠黑素瘤的生长有明显的抑制作用。B16-TM-SEA 肿

瘤疫苗免疫小鼠后,再用野生型 B16 细胞攻击,发现肿瘤的生长较 B16 疫苗、B16-SEA 疫苗免疫的小鼠显著减慢,而用 EL-4 细胞攻击,则肿瘤的生长与对照组无明显差异,且 B16-TM-SEA 疫苗免疫的小鼠其脾细胞 CTL 活性有明显增强。结果表明,使用 B16-TM-SEA 肿瘤疫苗免疫的 C57BL/6 小鼠,可以免受同种肿瘤细胞的攻击,可获得较强的特异性免疫保护作用。

本实验用跨膜型超抗原 TM-SEA 融合蛋白制备的肿瘤疫苗进行的抗肿瘤作用研究结果表明了这种制剂具有较显著的抗肿瘤作用。关于超抗原融合蛋白在抗肿瘤作用中的相关机制,作者课题组在前期的研究中已有过报道^[8~10],在本研究中,跨膜型超抗原 SEA 融合蛋白制备的肿瘤疫苗治疗的荷瘤小鼠的脾细胞 NK 和 CTL 活性增高,免疫后的小鼠其脾细胞 CTL 活性也明显增强,显示出是其中的一个抗肿瘤机制。跨膜型超抗原融合蛋白的研究,是一个将超抗原的免疫残基蛋白锚定在肿瘤细胞膜上的新方法,较单克隆抗体靶向的超抗原具有更加广泛的抗肿瘤作用和应用前景,为肿瘤的免疫治疗提供了一个新的思路。

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Antitumor effects of the fusion protein of transmembrane-staphylococcal enterotoxin A

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Abstract: **AIM** To prepare transmembrane-staphylococcal enterotoxin A fusion protein (TM-SEA) vaccine and to study its antitumor effect and the related mechanism. **METHODS** The B16 melanoma model of C57BL/6 mice was established, the antitumor effect and the protective immunity elicited by the B16-TM-SEA tumor vaccine were investigated, the natural killer(NK) and cytotoxicity T lymphocytes (CTL) activity of the splenocytes derived from the treated and immunized mice were measured by LDH release assay. **RESULTS** Tumor growth was inhibited and the survival time prolonged significantly in melanoma-bearing mice treated with the B16-TM-SEA tumor vaccine. The NK and CTL activity of splenocytes derived from the mice treated with the B16-TM-SEA vaccine increased significantly as compared with those in controls. Immunization of the mice

with the B16-TM-SEA vaccine elicited protective immunity against the following tumor challenge of B16 cells. Higher CTL activity was induced in vaccinated mice than that in unvaccinated mice. **CONCLUSION** The tumor vaccine based on TM-SEA fusion protein could elicit strong antitumor effect through more efficient induction of specific and nonspecific antitumor immune responses. This tumor vaccine may be as an efficient approach used for the treatment of cancer recurrence and micro-metastasis.

Key words: superantigen; transmembrane; fusion protein; immunotherapy; T lymphocytes

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