

人抑制素 β A 亚基片段的合成及抑制素 α 亚基 β A 亚基单克隆抗体的制备

孙 颖¹, 鲁桂琛^{1*}, 雷平生¹, 夏辉明², 高晓东², 黄 新²

(1. 中国医学科学院, 中国协和医科大学药物研究所, 北京 100050; 2. 河南医科大学病理生理研究室, 河南 郑州 450052)

摘要: 目的 合成人抑制素 α 亚基 1 个片段和 β A 亚基两个片段 β A(1-28) (GLECDGKVNICCKKQFFVSFKDIGWNDW), β A(82-114) (VPTKLRPMSMLYDDGQNIKKDIQNMIVEECG) 并制备了相应的单克隆抗体。方法应用固相多肽合成法人工合成了 1 个抑制素 α 亚基片段和 2 个 β A 亚基片段, 以化学合成的片段作为半抗原, 经抗原和单抗的制备获得 7 株杂交瘤细胞株。测定了单抗的滴度、特异性和相对亲和力。结果 单抗的鉴定及特异性分析实验表明这些单克隆抗体具有特异性、敏感性和实用性。结论 在这些实验的基础上建立测定血清抑制素水平的方法是可能的。

关键词: 抑制素; 固相多肽合成; 单克隆抗体; 免疫组化

中图分类号: R914.5; R967 文献标识码: A 文章编号: 0513 - 4870(2000)06 - 0426 - 05

抑制素^[1]是由性腺分泌的一种水溶性蛋白, 它由 α 和 β 亚基组成, β 亚基分为 β A 和 β B 亚基, α 和 β 亚基通过二硫键连接。抑制素有两种形式, 即抑制素 A 和抑制素 B, 抑制素 A 由 α 亚基和 β A 亚基组成, 抑制素 B 由 α 亚基和 β B 亚基组成。两个 β 亚基 (β A β A, β A β B, β B β B) 构成激活素(activin), 它的作用与抑制素相反。抑制素主要由卵巢颗粒细胞和睾丸滋养细胞(sertoli's cell)产生, 在精子的发育、卵泡的成熟和胚胎发育过程中起着重要作用, 从而调节生殖和发育过程。抑制素除抑制促卵泡释放激素(FSH)分泌外, 还在多种组织中起旁分泌和自分泌的作用。抑制素 α 亚基的 mRNA^[2]存在于大脑、垂体、脾、肾上腺等组织器官中, 且有广泛的生理活性^[3]。

抑制素在生殖系统的重要地位和它活性的广泛性, 为抑制素在临床应用开辟了广阔的前景^[4]。由于患有生殖系统肿瘤尤其是颗粒细胞瘤、粘液癌和滋养细胞瘤的患者血清抑制素水平异常增高。因此, 测定血清抑制素水平有可能作为原发和复发颗粒细胞肿瘤的一种指标^[5,6], 并可用于生殖系统肿瘤的早期诊断以及肿瘤的原发、复发、转移的诊断^[7]。将单克隆抗体联合使用^[8,9], 优选出特异性较好的单抗组合方式, 分别组装检测抑制素及其亚基的 ELISA 试剂盒, 可用于卵巢颗粒细胞肿瘤、粘液

癌和滋养细胞瘤的诊断。

因为 INH α (37-65) 片段在不同种属之间比较保守, 一级结构的一致性很强; α 亚基的片段选择主要是基于蛋白的首尾片段可能是抗原决定簇比较重要的区域^[10]。故本文应用固相多肽合成法人工合成了抑制素 α , β A 亚基的 3 个片段。并筛选出了针对 α , β A 亚基的 7 株杂交瘤细胞株, 鉴定了免疫学特性, 表明这些单克隆抗体具有特异性、敏感性和实用性。

3 个亚基的序列如下:

(1) INH α (37-65), 29 AA

Ile-Ser-Phe-Gln-Glu-Leu-Gly-Trp-Glu-Arg-Trp-Ile-Val-Tyr-Pro-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-Gly-Gly-Cys-Gly-Leu

(2) INH β A(1-28), 28 AA

Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp

(3) INH β A(82-114), 33 AA

Val-Pro-Thr-Lys-Leu-Arg-Pro-Met-Ser-Met-Leu-Tyr-Tyr-Asp-Asp-Gly-Gln-Asn-Ile-Ile-Lys-Lys-Asp-Ile-Gln-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly

实 验 部 分

试剂 各种氨基酸及树脂分别购自美国 Peninsula 实验室、德国 Fluka 和日本 Peptide

收稿日期: 1999-10-25

* 联系人 Tel: (010) 63165243, Fax: (010) 63017757, E-mail: gslu@public.bta.net.cn

Institute. Fmoc 保护氨基酸的侧链保护基分别如下: Thr(tBu), Ser(tBu), Asp(tBu), Lys(Boc), Arg(Pmc), His(Trt), Glu(tBu), Tyr(tBu), Cys(Trt)。Boc 保护氨基酸的侧链保护基分别如下: Asp(OcHex), Thr(Bzl), Lys(ClZ), Tyr(BrZ), Arg(Tos), Cys(4-MeBzl), His(Bom), Glu(OBzl)。Pam 树脂(自制, $0.38 \text{ mmol} \cdot \text{g}^{-1}$, 1% cross-linked, 100~200 mesh), Wang 树脂($0.85 \text{ mmol} \cdot \text{g}^{-1}$, 1% cross-linked, 100~200 mesh)。胎牛血清, 中国医学科学院血液研究所; DMEM 培养基, 美国 SCIENTIFIC 产品; 次黄嘌呤, 氨基嘌呤和丙酮酸钠, 德国 FLUKA; 8-氮杂鸟嘌呤, 美国 SERVE 产品; PEG1500, 荷兰进口分装; 羊抗鼠辣根过氧化物酶标记抗体, 中国军事医学科学院; 胸腺嘧啶核苷, 甲状腺球蛋白, 碳二亚胺, HEPES, 秋水仙素和降植烷, Sigma。

仪器 酶联免疫测定仪, 浙江永嘉分析仪器厂; CO_2 细胞培养箱, 日本岛津 WJ-IIK 型; 细胞培养板, 美国 COSTAR 产品。反向 C18 柱(北京分析仪器厂装填)、TSP 高效液相仪, Hitachi L-8500 型氨基酸分析仪, Autospec Ultima ETOF 型质谱仪。

1 抑制素片段肽的合成

采用固相多肽合成法, 应用 Boc 化学和 Fmoc 化学从 C 端逐步合成, Boc 化学采用 HF, anisole, thioanisole, EDT 等试剂裂解; Fmoc 化学采用苯酚, EDT, thioanisole, H_2O , TFA 等试剂裂解。合成的肽树脂经裂解得粗肽, 再经 HPLC 分离得到纯品。

产物经氨基酸分析及质谱鉴定。

2 抗原的制备

用甲状腺球蛋白(thyroglobulin, TG)作为载体, 以化学合成的半抗原与载体蛋白偶联形成完全抗原(H-TG), 偶联剂为 EDC; 或用 H-TG 与福氏佐剂(Freund's adjuvant)混合均匀而成免疫原, 以备免疫动物。

3 单抗的制备

免疫 BALB/C 小鼠(包括脾内注射快速免疫法); 细胞融合术; 单克隆抗体的检测与杂交瘤的筛选; 克隆化培养技术(包括有限稀释法); 单抗鉴定及特异性分析。

4 免疫组化的研究

以抗抑制素 α, β A 亚基的单抗在生殖系统与非生殖系统肿瘤中进行免疫组化的比较研究。

结果与讨论

Inhibin α (37-65)的合成见前文^[11], 本文补上质谱数据(ESI-MS: calculated: 3443.5, found: $1721.8 [M+2H]^{2+}$, $1148.5 [M+3H]^{3+}$, $861.4 [M+4H]^{4+}$)。两个 β A 亚基片段由于溶解度较差, 在分离纯化中损失较大, 收率较低。氨基酸分析数据和 HPLC 图谱见表 1, 图 1 和图 2。MS 数据(ESI-MS)为: INH β A(82-114): calculated: 3843.8, found: $1282.1 [M+3H]^{3+}$, $961.8 [M+4H]^{4+}$ 。

Tab 1 Amino acid composition analysis of INH β A and INH β A

| Peptide | Asp | Thr | Ser | Glu | Gly | Cys | Val | Met | Ile | Leu | Phe | Tyr | Lys | Trp | Arg | Pro |
|-----------------------|-------------|-------------|-------------|-------------|-------------|----------|-------------|----------|-------------|-------------|-------------|-------------|-------------|----------|-------------|-------------|
| INH β A(1-28) | 4.56 (5) | | 0.67 (1) | 2.00 (2) | 2.93 (3) | - (3) | 1.87 (2) | | 1.83 (2) | 1 (1) | 2.96 (3) | | 3.60 (4) | - (2) | | |
| INH β A(82-114) | 5.33 (5) | 0.87 (1) | 0.86 (1) | 4.19 (4) | 2.29 (2) | - (1) | 1.67 (2) | - (3) | 3.20 (4) | 1.80 (2) | | 1.73 (2) | 3.00 (3) | | 0.85 (1) | 1.84 (2) |

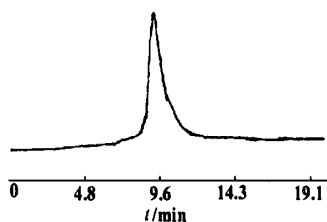


Fig 1 HPLC chromatogram of β A(1-28)
Gradient elution: MeOH/ H_2O (0.1% TFA) 50% ~ 80%, $1.0 \text{ mL} \cdot \text{min}^{-1}$, 30 min, 254 nm

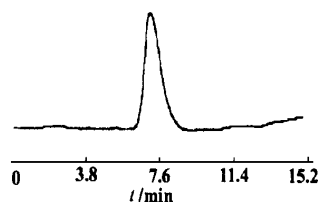


Fig 2 HPLC chromatogram of β A(82-114)
Gradient elution: MeOH/ H_2O (0.1% TFA) 65% ~ 100%, $1.0 \text{ mL} \cdot \text{min}^{-1}$, 20 min, 254 nm

INH α (37-65) 片段, INH β A(1-28) 和 β A(82-114) 片段的单抗研究结果见表 2, 表 3, 表 4 和表 5。

Tab 2 Result of McAb additivity test

| Cell line | A(492 nm) | AI + A2 | AI/ % | |
|-------------------|--------------|---------|-------|-------|
| α subunit | IA5 | 0.5 | | |
| | IV3C | 0.44 | | |
| | V5G | 0.87 | | |
| | IA5 + IV3C | 0.72 | 0.94 | 53.18 |
| | IA5 + V5G | 1.08 | 1.37 | 53.28 |
| | IV3C + V5G | 0.98 | 1.31 | 49.62 |
| β A subunit | III7B | 0.81 | | |
| | IV3G | 0.73 | | |
| | II3A | 0.62 | | |
| | III7B + IV3G | 1.02 | 1.54 | 32.47 |
| | III7B + II3A | 0.92 | 1.43 | 28.67 |
| | IV3G + II3A | 0.87 | 1.35 | 28.89 |

Tab 3 Detection of McAb relative affinity

| Cell line | 100 % | | 50 % | | Maximal combination | |
|-------------------|-------|----------|-------|----------|---------------------|--|
| | A | Dilution | A | Dilution | | |
| α subunit | IA5 | 1.78 | 1:800 | 0.80 | 1:5000 | |
| | IV3C | 1.56 | 1:200 | 0.78 | 1:1600 | |
| | V5G | 1.49 | 1:50 | 0.74 | 1:400 | |
| β A subunit | III7B | 0.95 | 1:200 | 0.49 | 1:800 | |
| | IV3G | 0.80 | 1:100 | 0.40 | 1:1600 | |
| | II3A | 0.92 | 1:50 | 0.45 | 1:400 | |
| | VI5C | 0.75 | 1:50 | 0.37 | 1:800 | |

Tab 4 Detection of McAb specificity

| Cell line | α (37-65) | β A (1-28) | β A (82-114) | Tg | FSH | LH | Others* |
|-----------|---------------------|---------------------|-----------------------|----|-----|----|---------|
| III7B | - | - | +++ | - | - | - | - |
| IV3G | - | - | ++ | - | - | - | - |
| II3A | - | - | + | - | - | - | - |
| VI5C | - | + | - | - | - | - | - |

* Others: AFP, Testosterone, CEA, progesterone, PRL, E₂. Symbols in the table show immunoreactive intensity: +++ intense, ++ subintense, + faintly, - negative

Tab 5 Determination of the titer of McAb

| Cell line | Titer | | | |
|-------------------|---------------------|---------------|----------------|--------|
| | Culture supernatant | Ascites fluid | PcAb antiserum | |
| α subunit | IA5 | 1:6000 | 1:100000 | 1:6400 |
| | IV3C | 1:1000 | 1:20000 | 1:1000 |
| | V5G | 1:400 | 1:1600 | 1:1000 |
| β A(82-114) | III7B | 1:400 | 1:64000 | 1:4000 |
| | IV3G | 1:600 | 1:16000 | 1:800 |
| | II3A | 1:400 | 1:8000 | 1:800 |
| β A(1-28) | VI5C | 1:200 | 1:4000 | 1:400 |

抗体特异性实验、相对亲和力测定、抗原竞争抑制实验、抗体的效价测定等证明了作者制备的单克隆抗体特异性强, 亲和性好。同时进行的免疫组化研究进一步证实了所得单抗的实用性。抑制素 α , β A 亚基的免疫组化结果见表 6 及表 7。

Tab 6 Immunohistochemistry of McAbs of Inhibin α subunit

| Sample | Age | Sex | Intensity of immunoreactivity | Sample number | |
|--------------------------------------|-----|--------|-------------------------------|---------------|---|
| Normal testis | | Male | Leydig's cells | +++ | 1 |
| | | | Theca cells | ++ | |
| | | | Spermatogonia | ++ | |
| | | | Spermatocytes | ++ | |
| Normal ovary | 32 | Female | Granulosa cells | +++ | 2 |
| | | | Theca cells | ++ | |
| | | | Lutein | 40 | |
| Left testis embryonal carcinoma | 53 | Male | ++ | 1 | |
| Testis spermatocytoma | 48 | Male | +++ | 1 | |
| Ovarian dysgerminoma | 63 | Female | - | 1 | |
| Ovarian thecoma | 44 | Female | - | 1 | |
| Ovarian embryonal carcinoma | 62 | Female | + | 1 | |
| Ovarian papillary cystadenocarcinoma | 67 | Female | + | 1 | |
| Ovarian granular cell tumor | 56 | Female | +++ | 1 | |
| Brain astrocytoma | 42 | Male | ++ | 1 | |
| Hydatidiform mole | 30 | Female | ++ | 1 | |
| Esophageal carcinoma | 56 | Female | - | 1 | |
| Prostate carcinoma | 60 | Male | - | 1 | |
| Stomach carcinoma | 48 | Male | +++ | 1 | |

Tab 7 Immunohistochemistry of McAbs of inhibin β A subunit

| Samples | Number | PBS buffer | McAb(1: 50) | McAb(1: 25) + porcine follicles 1NH(1: 4) preabsorbed at 37 °C for 2 h |
|-----------------------------|--------|------------|-------------|--|
| Sertoli's cells | | - * | ++ | - |
| Leydig's cells | | - | + | - |
| Spermatogonia | | - | + | - |
| Spermatocytes | | - | + | - |
| Spermatids | | - | - | - |
| Testis spermatocytoma | 1 | - | ++ | - |
| Testis embryonal carcinoma | 1 | - | + | - |
| Ovarian granular cells | 1 | - | +++ | - |
| Ovarian granular cell tumor | 1 | - | ++ | - |
| Ovarian dysgerminoma | 1 | - | - | - |
| Ovarian embryonal carcinoma | 1 | - | + | - |
| Cervix uteri tissues | 1 | - | - | - |
| Breast tissues | 1 | - | - | - |

* Symbols in the table show immunoreactive intensity: +++ intense, ++ subintense, + faintly, - negative

从表 6 和表 7 的结果可见,相关生殖系统肿瘤阳性,对照组将同样浓度的抗体与猪卵泡液中提取的抑制素共孵后再作为免疫组化的一抗,染色为阴性,说明反应是针对抗体的,这些结果进一步证实了人抑制素 α , β A 亚基的单克隆抗体的特异性、敏感性和实用性,为建立抑制素 A 的特异性的检测方法打下了基础。

致谢 氨基酸分析和质谱测定由本所分析室完成。

参考文献:

- [1] Ying SY, Inhibin, activin and follistatins[J]. *J Steroid Biochem*, 1989, **33**: 705.
- [2] Meunier H, Rivier C, Evans RM, et al. Gonadal and extragonadal expression of inhibin α , β A and β B subunits in various tissues predicts diverse functions[J]. *Proc Natl Acad Sci USA*, 1988, **85**: 247.
- [3] Robertson DN, Tsonis C, McLachlan RI, et al. Comparison of inhibin immunological and *in vitro* biological activities in human serum [J]. *J Clin Endocrinol Metab*, 1988, **67**: 438.
- [4] Risbridger GP, Robertson DM, De Kretser DM. Current perspectives of inhibin biology [J]. *Acta Endocrinol (Copenh)*, 1990, **122**: 673.
- [5] Burger HG. Inhibin as a tumor marker [J]. *Clin Endocrinol*, 1994, **41**: 151.
- [6] Cooke I, Ó'Brien M, Charnock FM, et al. Inhibin as a Marker for ovarian cancer [J]. *Br J Cancer*, 1995, **71**: 1046.
- [7] Burger HG. Clinical Utility of Inhibin Measurements [J]. *J Clin Endocrinol Metab*, 1993, **76**: 1391.
- [8] Baly DL, Allison DE, Krummen LA, et al. Development of a specific and sensitive two-site enzyme-linked immunosorbent assay for measurement of inhibin A in serum [J]. *Endocrinol*, 1993, **132**: 2099.
- [9] Groome NP, Illingworth PJ, Ó'Brien M, et al. Detection of dimeric inhibin throughout the human menstrual cycle by two-site enzyme immunoassay [J]. *Clin Endocrinol*, 1994, **40**: 717.
- [10] Saito S, Roche PC, McCormick DJ, et al. Synthetic peptide segments of inhibin α and β subunits: preparation and characterization of polyclonal antibodies [J]. *Endocrinol*, 1989, **125**: 2.
- [11] 孙颖, 鲁桂琛, 王德心, 等. 人抑制素 α 亚基片段的合成及活性研究 [J]. *药 学 学 报*, 1996, **31**: 106.

SYNTHESIS OF HUMAN INHIBIN β A FRAGMENTS, PREPARATION AND GENERATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN INHIBIN α AND β A SUBUNITS

SUN Ying¹, LU Gui-Shen¹, LEI Ping-Sheng¹, XIA Hu-Ming², GAO Xiao-Dong², HUANG Xin²

(1. Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China;

2. Pathophysiology Department of Henan Medical University, Zhengzhou 450052, China)

ABSTRACT: **AIM** To synthesize fragments of inhibin subunits β A(1-28) (GLECDGKVNICKKQFFVSFKDIGWNDW), β A(82-114) (VPTKLRPMSMLYYDDGQNIKKDIQNMIVEECG) and to obtain their monoclonal antibodies. **METHODS** The fragments of inhibin subunits α (37-65), β A(1-28) and β A(82-114) have been synthesized manually by stepwise solid phase procedure. These peptide products were conjugated with TG as antigens to prepare McAbs against inhibin α and β A subunit. After immunization, fusion, selection and cloning procedures, seven hybridoma cell lines were obtained. Ascites titer, specificity and relative affinity were identified. **RESULTS** These McAbs were shown to be highly special, sensitive and useful in detection tests. Initial immunohistochemistry test gave some important information for clinical utility. **CONCLUSION** The method of detecting serum inhibin level will be established on basis of the present research.

KEY WORDS: inhibin; solid phase peptide synthesis; monoclonal antibody; immunohistochemistry