应用Bhas 42细胞转化实验检测环磷酰胺、丝裂霉素C和氨苄西林钠致癌作用

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摘要：目的 采用Bhas 42细胞转化实验检测已知有遗传毒性的化学品在化学致癌中的引发和促生长作用，评价Bhas 42细胞转化实验检测化学品致癌作用的可靠性。**方法** ① 通过细胞毒性实验确定环磷酰胺、丝裂霉素C和氨苄西林钠进行Bhas 42细胞转化实验的浓度。② 引发实验：细胞接种当日为第0天，第1天换成含有相应最终浓度的受试物或0.5% DMSO的DF5F培养基，培养72 h，第4天换成不含药物的DF5F培养基，培养至第21天。第22天将细胞固定、染色、并计数细胞数大于50的集落数。③ 促生长实验：接种当日为第0天，第4天换成含有相应受试物或0.5% DMSO的DF5F培养基并连续培养至第14天，期间第7天，第11天更换同样培养基，每15天换成不含药物的DF5F培养基，培养至第21天。第22天固定、染色细胞、计数细胞数大于50的集落数。**结果** 按照70%的细胞存活率以及前期文献结果确定最终浓度为：氨苄西林钠1750 mg·L⁻¹；环磷酰胺1300 mg·L⁻¹；丝裂霉素C 0.01 mg·L⁻¹；3-甲基胆蒽1 mg·L⁻¹，佛波酯0.05 mg·L⁻¹。引发实验结果显示，3-甲基胆蒽，丝裂霉素C和环磷酰胺集落数显著多于空白对照、并且两者比值>2，判定为有引发作用的致癌化学品。促生长实验结果显示，佛波酯集落数显著多于空白对照、并且两者比值大于2，因此判定为有促生长作用的致癌化学品。**结论** Bhas42细胞转化实验不仅可以检测出遗传实验可检测出的致癌阳性化学品和非致癌化学品，还可检测出遗传实验结果为假阴性的致癌阳性化学品，可以作为一种快速易操作的致癌物预测体外模型。

关键词： Bhas42细胞；引发实验；促生长实验

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化学致癌物分为引发物和促癌物，部分具有遗传毒性的化合物可采用常规的遗传毒理实验检测。但根据文献报道，国际癌症研究机构归类为1，2A和2B级别的致癌物中有12%为非遗传致癌物[1]，常规的遗传毒理实验无法检测此类化学品，因此建立可用于非遗传致癌物检测的实验方法非常迫切及重要。

细胞转化实验是指对培养细胞诱发与肿瘤形成有关的表型改变，此种表型改变包括细胞形态、细胞生长能力、生化表型等变化，以及移植于动物体内形成肿瘤的能力等。1986年时日本学者Sasaki等[2]已经建立了BALB/c3T3二阶段细胞转化实验，该实验的两个阶段分别为引发阶段及促生长阶段。引发阶段即在培养皿中接种低浓度的细胞，在细胞进入分裂增殖期时加入受试物，在实验末将细胞固定染色，再通过检测细胞形态学上的改变来判断受试物的引发作用。而在促生长阶段则是在培养皿中接种细胞后先以N-甲基-N′-硝基-N-亚硝基胍为引发剂接触细胞，再接触受试物，在实验末将细胞固定染色，再通过检测细胞形态学的改变来判断受试物的促生长作用。

据文献报道，与动物体内致癌实验相比，在对184种化学物质的检测中，BALB/c3T3细胞转化实验能够检测出66%有机致癌物和87%无机致癌物，假阳性率为7.6%[3]。说明其与动物体内致癌实验相比，具有很好的一致性。Bhas 42细胞是转染了*v-Ha-ras*基因的BALB/c3T3细胞，正常情况下由于自身拥有敏感的接触抑制，在其覆盖满培养皿后会停止分裂增殖。若培养过程中接触致癌物质，则会失去细胞间的接触抑制并以异常的细胞形态最终形

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成集落。2004 年,Ohmori 等[4]提出单独使用 Bhas 42 细胞作为检测化合物的癌症促进能力的细胞转化实验;2005 年,Asada 等[5]改变了细胞接种浓度和受试物加入时间,从而建立 Bhas 42 二阶段细胞转化实验。其第一期与 BALB/c3T3 二阶段细胞转化实验中的第一期原理相似,但是在第二阶段中减少了引发物诱导的过程,直接接种接近细胞融合(near-confluent)浓度的细胞进行实验。

前期实验结果显示,Bhas 42 细胞在引发实验检测中已知的引发物 3-甲基胆蒽(3-methylcholanthrene, MCA)的实验结果为阳性,而已知的促癌物佛波酯(12-O-tetradecanoylphorbol-13-acetate, TPA)的作用为阴性;同时在促生长实验中 TPA 的实验结果为阳性,而 MCA 的实验结果为阴性[6]。本研究中选定 3 种化学品,采用 Bhas 42 转化细胞检测其引发及促生长作用,并与已知的遗传毒性实验结果进行对比,以进一步验证 Bhas 42 细胞转化实验对于致癌物质检测的准确性。

1 材料与方法

1.1 药物和试剂

EMEM 培养基,环磷酰胺(cyclophosphamide, CP), MCA 和 TPA 购自 Sigma 公司; DMEM/F12 培养基,胎牛血清(FBS)和胰酶(含0.25% EDTA)购自 Gibco 公司;丝裂霉素 C(mitomycin C, MMC)购自 Roche 公司;氨苄西林钠(ampicillin Na)购自华美生物工程有限公司; 二甲亚砜(DMSO)和吉姆萨色素购自国药集团化学试剂有限公司。

1.2 细胞及培养

Bhas42 细胞,购自 Japan Health Sciences Foundation, Health Science Research Resources Bank (PO JCRB0149);用含有 10% FBS 的 EMEM 培养基(M10F),5% CO_2,37℃恒温条件下培养;生长稳定后用含有 5% FBS 的 DMEM/F12 培养基(DF5F), 5% CO_2,37℃恒温条件下培养。

1.3 细胞毒性实验确定药物浓度

24 孔板,采用 DF5F 培养基将细胞密度调整至 2×10^7 L^{-1},每孔加入 0.5 ml 细胞悬液,接种当日为第 0 天。第 3 天换成含有受试物的培养基,每个浓度设置 3 个孔。第 7 天时使用 10% 甲醛固定 30 min,水洗,用 0.1 % 结晶紫染色 30 min,水洗后放入 37℃烘箱干燥。每孔加入 0.5 ml 提取液(0.9%柠檬酸钠+盐酸 0.02 $mol\cdot L^{-1}$ +50%乙醇),于波长 540 nm 处读取的各组吸光度值,各浓度组与空白对照组的吸光度的百分比值代表细胞存活率。本实验选取存活率为 70% 的浓度作为实验中各受试物的最终浓度,当存活率未出现明显下降时,则选取细胞毒性实验中的最高浓度。

1.4 引发实验

用 DF5F 培养基调细胞密度为 4×10^6 L^{-1},每孔加入 2 ml 细胞悬液,每个受试物采用一块 6 孔板,并平行设置空白对照,接种当日为第 0 天。第 1 天,将培养基换成含有相应受试物的 DF5F 培养基,空白对照板换成含有 0.5% DMSO 的 DF5F 培养基(DF5F 中 DMSO 的比例与受试物组相同)。第 4 天,换为 DF5F 培养基并持续培养至第 21 天,其中第 7 天,第 11 天,第 14 天更换培养基。第 22 天时,甲醇固定细胞 10 min 后,用5% 吉姆萨色素溶液染色 30 min,计数细胞集落数。

1.5 促生长实验

用 DF5F 培养基调细胞悬液密度 2×10^7 L^{-1},每孔加入 2 ml 细胞悬液,每个受试物采用一块 6 孔板,并平行设置空白对照,接种当日为第 0 天。第 4 天,将培养基换成含有相应受试物的 DF5F 培养基,空白对照板换成含有 0.5% DMSO 的 DF5F 培养基(DF5F 中 DMSO 的比例与受试物组相同)连续培养至第 14 天,其中第 7 天,第 11 天更换含有受试物或 0.5% DMSO 的 DF5F 培养基。第 15 天换为 DF5F 培养基并持续培养至第 21 天。第 22 天时,甲醇固定细胞 10 min 后,用 5% 吉姆萨色素溶液染色 30 min,计数细胞集落数。

1.6 引发和促生长实验结果判断及评价标准

根据集落的嗜碱性程度、细胞的密度及多层性、集落边缘细胞的方向对转化后的细胞进行评价,大于 50 个细胞才可记为一个集落。计数集落个数后采用单侧 Dunnett t 检验进行统计[7]:① 受试物组集落数多于空白对照组,且在单侧 Dunnett t 检验中 $P<0.05$;② 受试物组与空白对照组的集落数平均值比值大于 2。同时符合上述中的两条即可判断为阳性(+),仅符合第一条为可疑结果(±),否则判断为阴性(-)。

2 结果

2.1 环磷酰胺、丝裂霉素 C 和氨苄西林钠对细胞存活率的影响

根据细胞毒性实验结果(图 1A, B, C),CP、MMC 和氨苄西林钠正式实验的终浓度分别为 1750, 1300 和 0.01 $mg\cdot L^{-1}$。根据文献报道及前期实验结果,引发物 MCA 实验终浓度为 1 $mg\cdot L^{-1}$,促癌物 TPA 实验终浓度为 0.05 $mg\cdot L^{-1}$。

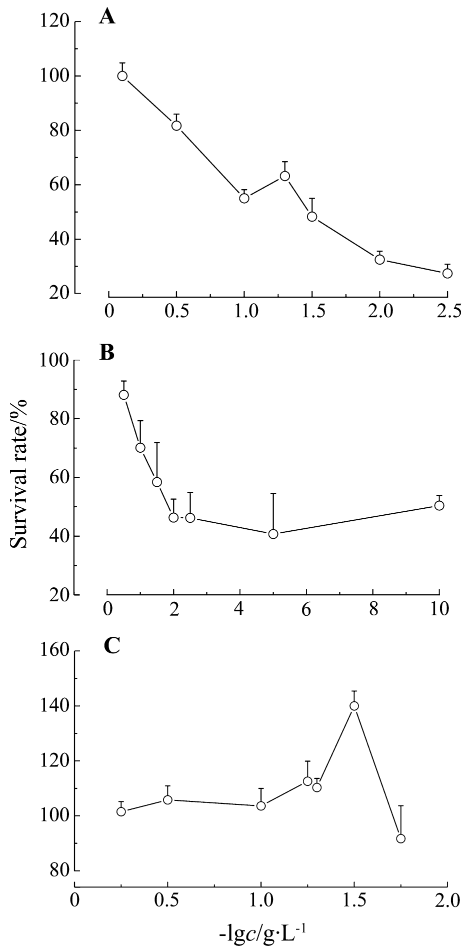

Fig. 1 Effect of cyclophosphamide (A), mitomycin C (B) and ampicillin Na (C) on cell survical. The cells incubated with drugs for 4 d.

2.2 环磷酰胺、丝裂霉素 C 和氨苄西林钠对集落数的影响

引发实验结果显示(表 1),与空白对照组相比,化学品 MCA, MMC 及 CP 的集落平均数大于空白对照组的 2 倍,且统计结果显示有显著性差异($P<0.05$),根据 **1.6** 项中的判定标准,MCA, MMC 及 CP 为具有引发作用的致癌化学品。与空白对照组相比,氨苄西林钠的集落平均数小于空白对照组的 2 倍,且统计结果显示无显著性差异,按照判定标准说明氨苄西林钠不具有引发作用。

促生长实验结果显示(表 2),与空白对照组相比,化学品氨苄西林钠、MMC 及 CP 的集落平均数小于空白对照组的 2 倍,且统计结果显示无显著性差异,根据 **1.6** 项中的判定标准,氨苄西林钠、MMC 及 CP 不具有促生长作用。与空白对照组相比,化学品 TPA 的集落平均数大于空白对照组的 2 倍,且统计结果显示有显著性差异($P<0.05$),根据**1.6** 项中的判定标准 TPA 为具有促生长作用的致癌化学品。

Tab. 1 Effect of cyclophosphamide (CP), mitomycin C (MMC) and ampicillin Na on foci number in Bhas 42 cell initiation assay

Drug/mg·L^{-1}	Foci numbera	Ratio	Judgment
Blank control	4.2 ± 0.4	NA	NA
MCA 1	47.0 ± 4.5*	11.2	+
MMC 0.01	32.5 ± 5.4*	7.7	+
CP 1300	43.3 ± 9.5*	10.3	+
Ampicillin Na 1750	5.7 ± 0.5	1.4	–

The day of seeding cells was defined as the day before drug. On the 1st day, medium in each well was changed with the medium DF5F containing test article or 0.5% DMSO, and the treatment in the initiation phase was continued for 72 h. Following the exposure period, all treatment media were removed and the cells were refed with medium without the test article (4th day) and subsequently cultured in DF5F until the 21st day, receiving medium exchanges on the 7th, the 11th and 14th days. The cells were fixed and stained on the 22nd day and counted the foci whose cells number was more than 50. Judgment: ① a significant increase ($P<0.05$) in a one-sided Dunnett t test; and ② more than a two-fold increase as compared with the blank control. Chemicals which satisfied these two criteria were judged to be positive (+). Chemicals which met only the first criterion, but not the second, were considered equivocal (±). Ratio = foci number of test article/foci number of blank control; NA: not available. $\bar{x} \pm s$, $n=6$. * $P<0.05$, compared with blank control group.

Tab. 2 Effect of CP, MMC and ampicillin Na on foci number in Bhas 42 cell promotion assay

Drug/mg·L^{-1}	Foci number	Ratio	Judgment
Blank control	12.0 ± 1.8	NA	NA
TPA 0.05	43.0 ± 4.3*	3.6	+
MMC 0.01	11.7 ± 2.2	1.0	–
CP 1300	14.2 ± 2.2	1.2	–
Ampicillin Na 1750	15.0 ± 2.3	1.3	–

TPA: 12-O-tetradecanoylphorbol-13-acetate. The day of seeding cells was defined as the day before drug. On 4th day, medium in each well was changed with the medium DF5F containing test article or 0.5% DMSO, and the treatment in the promotion phase was continued to 14th day. During the exposure period, all treatment media were replaced with medium containing the test article or 0.5% DMSO on the 7th and the 11th days. The cells were then subsequently cultured in the DF5F without the test article from the 15th to the 21st day. The cells were fixed and stained on the 22nd day and counted the foci whose cells number was more than 50. The following criteria were used for the evaluation of transformation results: ① a significant increase in a one-sided Dunnett test, with a significance level of 5% ($P<0.05$); and ② more than a two-fold increase as compared with the solvent control. Chemicals which satisfied these two criteria were judged to be positive (+). Chemicals which met only the first criterion, but not the second, were considered equivocal (±). Ratio = foci number of test article/foci number of blank control; NA: not available. $\bar{x} \pm s$, $n=6$. * $P<0.05$, compared with blank control group.

与这些药物已知的 Ames 检测[3]、ML[3]、体内外染色体畸变[3]和 MN[3]结果相比对(表 3)发现,Bhas 42细胞转化实验不仅可以检测出以上实验

Tab. 3 Comparison of results in the Bhas 42 cell transformation assay with those in genotoxicity assays

Drug	Bhas 42 cell transformation assay		Ames [8]	ML[8]	Chromosome aberration (rodent and human cells) [8]		MN[8]
	Initiation assay	Promotion assay			*In vitro*	*In vivo*	
MCA	+	−	+, +	+	−, −	NA	NA
TPA	−	+	−	NA	NA	NA	NA
MMC	+	−	+ (CCRIS)	+, +	+, +	+, +, +	+, +, +
CP	+	−	+, +/−, +/−	+, +, +, +	+, +	+, +/−	+, +, +
Ampicillin Na	−	−	−	−	−	−	−

NA: not available based on CCRIS. ML: mouse lymphoma test, MN: micronucleus test; CCRIS: the chemical carcinogenesis research information system. "+" means "positive in one database", "+, +" means "positive in two different databases", "+, +, +" means "positive in three different databases", "+, +, +, +" means "positive in four different databases", "+/−" means that there are diverging results inside a database, "−" means negative results. The databases used in this table are: NTP, GENETOX, CCRIS and IUCLID.

中阳性结果的化学品如 MCA, MMC 和 CP 以及阴性结果的化学品(如氨苄西林钠),并可检测出Ames结果为阴性而实际有致癌风险的化学品(如 TPA),根据文献报道 TPA 是已知的可导致小鼠皮肤癌的促癌物[8-9]。根据本实验的结果,Bhas 42 细胞对化学品致癌作用的判定与国际癌症研究中心对致癌物质的分类结果一致。

3 讨论

通过本实验结果可知,Bhas 42 细胞转化实验对于化学品的致癌作用检测结果准确性较高,是一种成本低、周期短、化学品消耗少且能够检测非遗传性致癌化学品的体外替代模型。在药物研发早期可以用于检测化学品致癌性从而进行早期筛选,在进行化学品致癌作用机制研究时也可用于化学品致癌作用的类型分析和判断。目前已有日本学者采用DNA 微阵列和实时 RT-PCR 技术,通过细胞转化实验筛选出 22 个相关基因(这些基因全部与细胞分裂周期、调节转录、抗凋亡、正性调节细胞增殖有关),并认为这 22 个基因标志物可作为检测促癌剂的一个有效工具[10],这也是基于体外筛选模型对致癌作用机制工作的进一步探索。

当然,在致癌评价中哺乳动物长期致癌实验仍是经典实验方法。体外转化实验的终点仍属形态转化或恶性前期转化,此种转化可能发展为真正的肿瘤,也可能停滞在此阶段,不继续恶化。因此对体外转化实验阳性结果仅提示受试物有致癌可能性。作为体外实验检测体系,Bhas 42 细胞转化实验还需要更进一步的验证后才能被广泛认可,今后可联合多个实验室针对多种化学品进行联合验证以取得更多的数据。这将是一个长期而艰巨但是充满希望的工作。

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Carcinogenesis activity detection of cyclophosphamide, mitomycin C and ampicillin Na by cell transformation assay in Bhas 42 cells

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Abstract: OBJECTIVE To detect the initiating and promoting carcinogenicity activity of some genotoxic chemicals, to evaluate the performance of Bhas 42 cell transformation assay for the detection of chemical carcinogenicity. **METHODS** ① The dose of cyclophosphamide(CP), mitomycin C (MMC) and ampicillin Na applicable to the Bhas 42 cell transformation assay by cytotoxicity test was defined. ② Initiation assay: the day of seeding cells was defined as the day before drug, on the 1st day, medium in each well was changed with the medium DF5F containing test chemical or 0.5% DMSO, and the treatment in the initiation phase was continued for 72 h. Following the exposure period, all treatment media were removed and the cells were refed with medium without the test chemical (the 4th day) and subsequently cultured in DF5F until the 21st day, receiving medium exchanges at the 7th day, the 11th day and the 14th day. The cells were fixed and stained on the 22nd day and counted the foci whose cells number was more than 50. ③ Promotion assay: the day of seeding cells was defined as the day before drug. On the 4th day, medium in each well was changed with the medium DF5F containing test chemical or 0.5% DMSO, and the treatment in the promotion phase was continued to the 14th day. During the exposure period, all treatment media were replaced with medium containing the test chemical or 0.5% DMSO on the 7th day and the 11th day. The cells were then subsequently cultured in the DF5F without the test chemical from the 15th day to the 21st day. The cells were fixed and stained on the 22nd day and counted the foci whose cells number was more than 50. **RESULTS** According to the results of cytotoxicity test and previous results, the final concentration of chemicals were ampicillin Na: 1750, CP: 1300, MMC: 0.01, 3-methyl-cholanthrine (MCA): 1 and 12-O-tetradecamoylphorbol-13acetate (TPA): 0.05 $mg \cdot L^{-1}$. The initiation assay results showed as the foci numbers of MCA, MMC and CP were significant increased in *t*-test and more than a 2-fold increase as compared with the solvent control, the above chemicals were the initiation positive. The promotion assay results showed as the foci number of TPA was significantly increased in *t*-test and more than a 2-fold increase as compared with the solvent control, TPA was the promotion positive. **CONCLUSION** Bhas 42 cell transformation assay can detect not only the positive and negative chemicals but also the false-negative carcinogens. The Bhas 42 cell transformation assay can be used to detect the initiating and promoting carcinogenicity activity of chemicals.

Key words: Bhas 42 cell line; initiation; promotion

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