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提升私校研發能量專案計畫—腐植酸及重金屬的環境化學
與環境毒物學研究--子計畫四:腐植酸對砷促癌作用之影響
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腐植酸及重金屬的環境化學與環境毒物學研究

子計劃四

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前言

腐植酸 (humic acid) 是存在於環境中的酚類聚合物 (polymers of phenolic acids)。近 40 年來，中國大陸和台灣學者在「腐植酸自由基」與克山病、大骨節病、甲狀腺腫和烏腳病的致病關係的研究，證實飲用水和土壤中過高的腐植酸有利於上述地方性疾病的發生。烏腳病是台灣西南沿海地區特有的一種末梢血管疾病，發病初期，患者通常是四肢先有麻木及冰冷現象，接著患部潰爛，然後壞疽病變。至今仍無良好預防及治療方法，唯有以手術鋸去患肢，但症狀仍會繼續惡化。此外，烏腳病地區亦具有高度流行率的癌症 (膀胱癌、腎臟癌、肺癌、皮膚癌、肝癌、攝護腺癌等)。過去一直認為致病因子是砷，然目前已有很多報告指出腐植酸所產生細胞內氧化壓力會造成血栓與血球破壞是烏腳病之致病因之一，又腐植酸會產生氧化性 DNA 與癌症之發生似乎有所關聯，然腐植酸與砷在癌化上關係如何，又在促癌化上所扮演角色如何？有待進一步之研究。

在過去研究中指出飲水中或環境中無機砷的量與皮膚癌，肺癌及膀胱癌有密切關聯性，然其機制不甚清楚。有學者指出 arsenic 是一種 co-mutagen 或 co-carcinogen，另有學者指出無機砷可促進轉譯因子 NFkB 及 AP-1 之活化進而促進癌化。台灣學者先後發現井水中砷含量偏高和腐植酸偏高，且烏腳病好發區也有高癌症發生率的現象，因此腐植酸與砷兩者對於癌症之發生與兩者關係有待進一步了解。

多重步驟癌化過程主要分成三階段: initiation, promotion, progression，又細胞癌化初期會有細胞轉型之現象，由於過去文獻指出細胞 transformation 初期會有細胞骨架改變，細胞遷移能力增強等現象，長期則會有促進 anchorage independent growth，例如 tumor promoter :TPA 可造成 JB6(+) cell 細胞骨架改變及促進 cell migration，促進 anchorage independent growth。故本研究利用一 JB6(+) cell line 來評估腐植酸與砷在促癌化上之作用。

本研究首先評估腐植酸對砷促癌作用之影響，首先比較有無腐植酸之促癌作

用能力，皆著觀察有無腐植酸對無機砷(III)促進 JB6(+)細胞轉型能力之影響。目前在細胞實驗中發現腐植酸本身可增強 JB6+細胞內自由基(ROS)之量，又處理過腐植酸之 JB6(+)細胞會增強細胞內 oxidative status，並改變細胞形態及 migration 能力，在較長期之培養下也發現其會造成細胞轉形及促進 anchorage independent growth，因此腐植酸具有 tumor promotion 之能力，另外動物實驗發現腐植酸具有促進小老鼠皮膚上皮增生之現象。又發現腐植酸可增強無機砷(As_2O_3) migration 及 transformation 之能力，其作用與增強 JNK 訊息路徑與活化 AP-1 及 NFkB 有關。

研究目的

透過上皮細胞株之研究了解腐植酸在皮膚癌發生中所扮演角色,以及腐植酸與砷兩者在癌症發生中兩者關係如何?

研究方法

(一) 腐植酸 (Humic acid; HA) 純化步驟

量取適量之 humic acid (Aldrich) 粉末溶於 1N NaOH (in dd H₂O)，PH=10,攪拌至溶解後靜置 2-3 小時，並不時攪拌後，加入 1NHCl (in ddH₂O)，調 PH 到約為 2，產生沉澱,靜置隔夜，分裝到 50ml 試管，以 3000 rpm 15°C，離心 10 分鐘去掉上層黃色透明液體，將下層褐色沉澱物收集，重複上述步驟 鹼→酸→離心→分離 共 3 次,收集最後一次沉澱物後先以較少量之 1N NaOH 攪拌使之溶解後再加入少許 dd H₂O 攪拌，調整至 pH 約為 7(視需要可加入少許 1N HCl 調整 pH 值)將 HA 純化後之溶液，以濾紙過濾去除不溶性雜質,將溶液冷凍乾燥後收集粉末置於乾燥箱內備用。

(二) 細胞試驗

(A) JB6 (+) 上皮細胞 (JB6 clone 41 cell) 之培養

JB6 (+) 細胞是一 post-initiated mouse epidermal cell line 很常用於 tumor promotion 之研究。將 JB6 (+) 細胞培養於 5 % FBS 之 MEM (內含 100U/ml penicillin; 100 μ g/ml streptomycin; 2 mM L-glutamine) 的角瓶中，置於 37 °C，5% CO₂ 培養箱中培養，長滿後以 PBS wash 2 次，加以 1 ml trypsin-EDTA，將細胞打下，立即加入 10 ml MEM 放入 15 ml 離心管離心去上液，再加新 medium 重新培養。

(B) 轉形作用試驗

根據 Mondal 等人之方法(Ref), 種植 200 個 JB6 細胞於 60 mm dish, 培養 24 h 後加入各種試藥, 1 天後加入新 medium 每週換兩次 medium 培養 6-8 週後以酒精固定, 利用 5 % Giemsa 染色 30 分鐘, 顯微鏡下觀察計算 3 mm 之 foci 染色較深者為 type II 及 type III 計算 foci 數目。

(C) 細胞外型之偵測

將細胞以腐植酸, As₂O₃ 或同時處理兩者 48 後再以 PBS wash 兩次後再培養兩天後 trypsin-EDTA 將細胞由培養皿 trypsinize 下來, 用 PBS 沖洗後, 以 DAPI 染核, DAPI 染色 1h (DAPI (4',6-Diamidino-2-phenylindole) 是一種 DNA 染劑, 可以穿透細胞膜至細胞核內, 插入雙股 DNA 的 minor groove 中 Adenine 及 Thymine rich 的位置。利用 DAPI 在螢光顯微鏡 (340 / 380 excitation) 可以看出 DNA 的改變, 來判斷是否有別于正常細胞)。以 PBS wash 2X。再以相位差顯微鏡進行觀察。

(D) Wound healing method

細胞培養換 starvation medium (含 0.1% FBS) 48 小時後, 加入不同濃度的腐植酸及氧化砷在含血清之培養基中處理 48 小時後, 劃線, 換 starvation medium 再繼續培養 48 小時, 以相位差顯微鏡觀測後照像, 並任選五個視野數細胞數目平均後作圖。

(F) Anchorage-independent growth assay

將細胞以腐植酸及氧化砷處理 48 h 再以 PBS wash 兩次後算取 5000 個細胞種於 soft agar dish (上層: 1ml 0.33% Basal Medium Eagle(BME)含 10% FBS, 下層: 3.5 ml 0.5% BEM) 將細胞培養於 95 % air plus 5% CO₂, 37°C, 21 天後，顯微鏡下觀察並計算多於 8 細胞之 colony.

(H) 西方墨點法(Western blot analysis)：

實驗原理為利用電流將帶有負電荷的不同分子量的蛋白分離，並以專一抗體與標的蛋白結合，放大並顯示該蛋白表現的變化情形。

試劑：

1. Lysis Buffer 內含 RIPA buffer：150mM NaCl，1% NP-40，0.5% Deoxycholic acid，0.1% SDS，50mM Tris-base，PH=7.5；1mM sodium orthovanadate；100µg/mL PMSF；170µg/mL Leupeptin。
2. Sample Buffer 內含 2mL 0.5M Tris-HCl (PH=6.8)；1.6mL Glycerol；2mL 10% SDS；0.8mL 2-β-mercaptoethanol；0.4mL 0.5% Bromophenolblue
3. Washing buffer 內含 PBS with 0.5% Tween-20

步驟：

(1) 細胞的準備與處理

將細胞培養後，以 trypsin-EDTA 將細胞由培養皿 trypsinize 下來，用 PBS 沖洗後以 1000rpm 離心 5 分鐘，取沈澱細胞，加培養基使細胞懸浮，將細胞濃度調整成 1×10^6 cells/10 cm dish，培養於 10 cm dish 中。待細胞貼壁後，以 0.1% MEM 進行 Starvation overnight。然後更換新的培養基再依序將藥物

加入(As_2O_3 先前處理 5 分鐘，再加入 Humic Acid)，培養至特定時間在將細胞收下。

(2) 蛋白質的萃取及定量

將舊的培養基倒掉，利用 cold PBS wash 2 次，在利用刮杓將細胞刮下收集至 eppendorf 離心 1000rpm 5 分鐘，再去除 PBS，然後加入 Lysis buffer 50 μ l (內含 proteinase inhibitor) 加入放置於 4 $^{\circ}C$ 震盪 30 分鐘，再離心 10000g 10 分鐘，取上清液即為 Total cell lysate，再以 Coomassie blue 定量蛋白質含量(以 U-2000 Spectrophotometer 分析吸光值，來測定各標本之蛋白質量)。

(3) SDS 膠體之準備

將電泳玻璃片洗淨，擦拭乾淨後，把兩片玻璃架至電泳座上固定，若欲分離小分子量之蛋白，則配置 12% 的下層 separating gel；若欲分離大分子量之蛋白，則配置 8% 或 10% 的下層 separating gel。配膠所需物質混合均勻後，直到液面離 well 約 1.5 cm 處為止，加入二次水覆蓋液面，待膠體凝固後，將上層的二次水吸乾，再配置上層 stacking gel 倒入及插入 comb，待完全聚合後，小心將 comb 拔出，再將 sandwich clamp assemblies 放入電泳槽中，加入 1X Running buffer 觀察無洩漏情況後，即完成膠體與電泳槽之製備。

(4) 加入樣本 (Loading sample) 及電泳

樣品槽由左至右，依序注入 protein standard marker 及 sample 以進行電泳。電壓在前十分鐘(stacking gel)用 80 volts，帶 sample 移動到 separating gel 再調高到 120 volts。待 tracking dye 跑至接近底層 1cm 處並且 marker 可看到清楚的分開，此時關掉電源，結束電泳，隨即拆除電泳儀組，進行轉印 (electrotransfer) 步驟。

(5) 轉漬 (electrotransfer) 步驟

先將 nitrocellulose (NC) paper (transfer membrane) 及 3M 濾紙浸泡於轉液中，直到紙之各部分均勻潤濕為止。Electrotransfer 的方向是由負極 (anode) 到正極 (cathode)，而 NC paper 要置於接近正極，膠體那一面向負極。將已浸濕的 3M 濾紙，整齊重疊至於半乾式電轉漬器中間，為最底層。再平鋪膠體於濾紙之上，並將 NC paper 蓋在膠體上，最上層再鋪上 3M 濾紙，最後蓋上蓋子。接上電源，維持電壓 100 volts，轉漬 1 小時。

(6) 免疫轉漬法 (immunoblotting)

轉漬完成後，將 NC paper 置於 5% blocking solution 中，用振盪器振盪室溫 1 小時 blocking。以 washing buffer 洗三次再加入初級抗體，用振盪器振盪 overnight at 4°C。隔天之後將初級抗體吸起以 washing buffer 洗三次，再加入二級抗體用振盪器振盪室溫 1 小時之後將二級抗體吸起以 washing buffer 洗三次，將 transfer membrane 夾起蔭乾，置入 chemiluminescence reagents (PerkinElmer Life Science ; Western Lightning : Enhanced luminol reagent 1.5ml 配上 Oxidizing reagent 1.5ml) 作用反應螢光。最後以冷光儀偵測觀察結果。

(三) 動物實驗

加藥方式：

分別於每週一、三、五加藥。

Control (6 隻)：投予 Acetone 200 μ l/mouse 每週 3 次，持續 24 週。

B[a]p single dose (6 隻)：只在第 1 週投予 0.2 μ mol /200 μ l acetone，其餘 23 週只給予 acetone。

B[a]p+HA_H (6 隻)：只在第 1 週投予 0.2 μ mol B[a]p /200 μ l acetone，其

餘 23 週給予 $250 \mu\text{g HA}_H / 200 \mu\text{l acetone}$ 。

B[a]p+HA_L (6 隻)：只在第 1 週投予 $0.2 \mu\text{mol B[a]p} / 200 \mu\text{l acetone}$ ，

其餘 23 週給予 $50 \mu\text{g HA}_L / 200 \mu\text{l acetone}$ 。

HA_H (6 隻)：每週給予 $250 \mu\text{g HA}_H / 200 \mu\text{l acetone}$ 。

處理方式：

於實驗後第 24 週犧牲老鼠，取其加藥皮膚 (2/3 送至病理切片室；1/3 用來 test western)。

結果與討論

1. 在相位差顯微鏡下觀察發現 HA 或 As_2O_3 處理過之細胞變為較細長而原細胞較圓，又 HA 和 As_2O_3 一起處理其變化與處理 TPA (tumor promoter) 者有類似之結果(Fig.1)，然而不管處理 HA 或 As_2O_3 或 TPA 或 HA 加 As_2O_3 對核皆無明顯影響。因此 HA 可能具有增強 As_2O_3 tumor promotion 之能力。
2. mouse skin JB6 Cl41 cell 是一對 TPA 誘發 neoplastic transformation 敏感之細胞，根據文獻指出轉形細胞前期有較強之 migration activity (ref)，因此我們測定了 HA 或 As_2O_3 或 HA 和 As_2O_3 處理 48 h 後再 culture 2 天之 JB6+ cell 其 migration activity, 由結果中發現 10 and 100 $\mu\text{g/ml}$ 劑量之 HA 或 As_2O_3 皆有意義的增加 cells migration (Fig.2) 且 HA 會促進 As_2O_3 所誘發的 migration。
3. 正常細胞常以 monolayer 生長, 當細胞發生轉型或癌化時則會重疊的生長，在 Fig.3 中發現 HA 加入會增加 As_2O_3 所誘發的 foci formation，另外 anchorage-independent growth 是細胞轉型之一特性, HA 或 As_2O_3 單獨處理皆有促進細胞 anchorage-independent growth (Fig.4)，而 HA 和 As_2O_3 一起處理有加強此作用，因此 HA 具有增強 As_2O_3 tumor promotion 之能力。
4. 過去研究顯示 TPA 誘發 mouse skin JB6 Cl41 cell 細胞轉型與活化 MAPKs 訊息路徑有關. MAPKs 包括 Erk, JNK, p38, 在我們研究中發現 HA 主要會活化 Erk, p38, TPA 和 As_2O_3 則三者皆會活化，而 HA 會加強 As_2O_3 所誘發 JNK 的活化(Fig.6), 另外發現其會促進核中 p-cjun 及 p65 量(Fig.7)，亦即會促進 AP-1 及 NFkB 之轉譯能力。
5. 另外由於 HA 會加強 As_2O_3 所造成形態的改變，我們進一步觀察 cell membrane vinculin 及 connexin 43 之表現，結果發現 HA 會減少 As_2O_3

所造成 connexin 43 之表現即減少細胞與細胞之 communication, 即有促進細胞轉型之作用.

6. 由以上結果我們發現 HA 及 As₂O₃ 皆有 tumor promotion 之作用且 HA 具有增強 As₂O₃ tumor promotion 之能力。然而我們在 mouse skin two step carcinogenesis 模式中, 並無法成功誘導 skin tumor 之發生. 在 Fig.9 中僅成功發現 HA 會誘導增生之現象, 且發現其對 skin 蛋白的影響與 TPA 有類似之處, 如促進 iNOS, Cox2, ODC, PCNA, OPN 等之表現(Fig.10).
7. 由以上結果我們發現 HA 及 As₂O₃ 皆有 tumor promotion 之作用且 HA 具有增強 As₂O₃ tumor promotion 之能力, 因此 HA 可能會提高 As₂O₃ 污染地區癌症之發生率及嚴重性, 值得重視.

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計劃成果自評

本計劃已發表一篇文章,另一篇正積極籌備中,但未能成功建立腐植酸及砷之皮膚癌動物模式,然而發現腐植酸有促進砷誘發細胞轉形之能力及其作用機制.

遺憾是腐植酸是否有促進砷誘發其它癌症(如肺癌及肝癌)的相關作用及機制計劃無法如願拿到獎助,這方面相關計劃恐就此打住。希望以後有經費與機會做進一步探討.

已發表文獻

Lu FJ, **Tseng TH***, Lee WJ, Yen CC, Yin YF, Liao CW, Liu KM (2006) Promoting neoplastic transformation of humic acid in mouse epidermal JB6 Cl41 cells. **Chemico Biological Interactions**, 162, 249-258.

圖 表

Fig.1

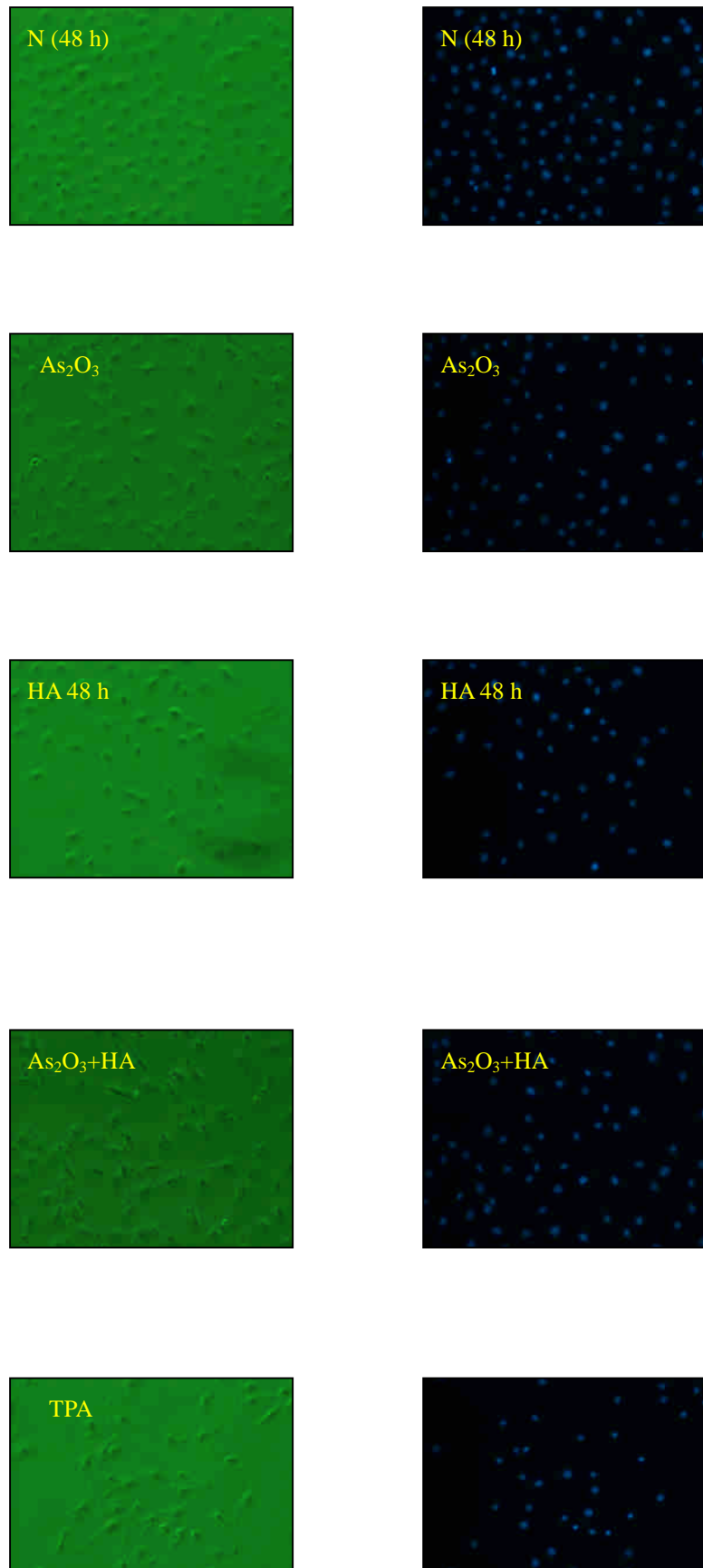
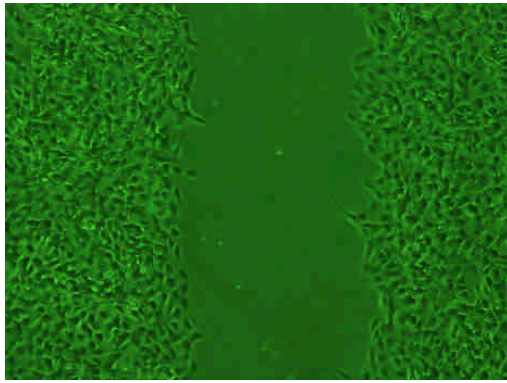
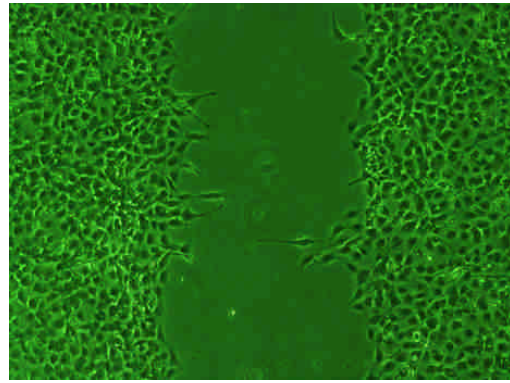


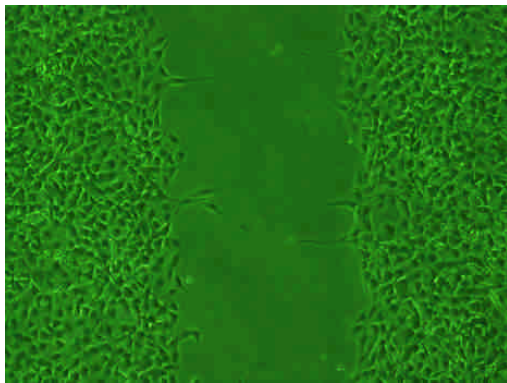
Fig. 1. Effect of HA and arsenic oxide on the morphology of JB6 Cl41 cells. The cells were treated with 20 ng/ml TPA, HA, arsenic oxide or HA and arsenic oxide co-administration for 48 h. morphology of cells was observed by phase contrast microscopy (200×)(left column). The nuclear morphology was analyzed by DAPI nuclear staining, which displayed *blue* fluorescence (middle column).



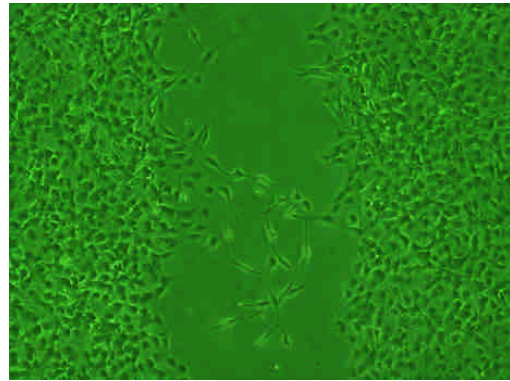
Normal



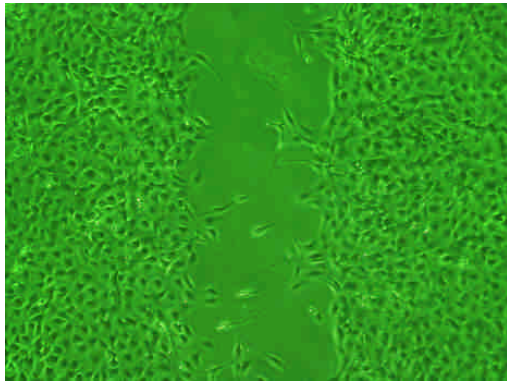
As_2O_3 (2 μ M)



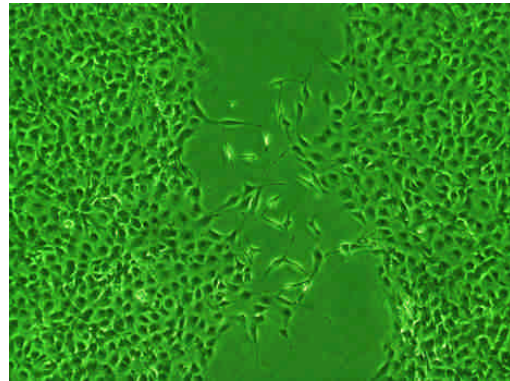
HA 1 μ g/mL



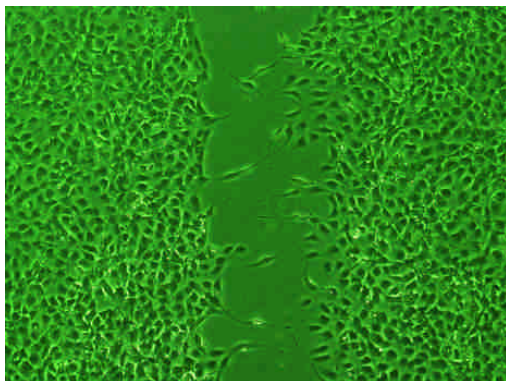
As_2O_3 2 μ M + HA 1 μ g/mL



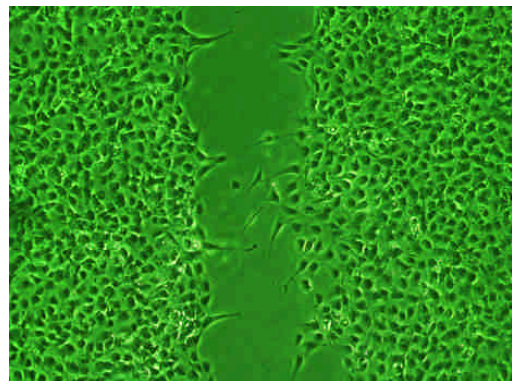
HA 10 μ g/mL



As_2O_3 2 μ M + HA 10 μ g/mL



HA 100 μ g/mL



As_2O_3 2 μ M + HA 100 μ g/mL

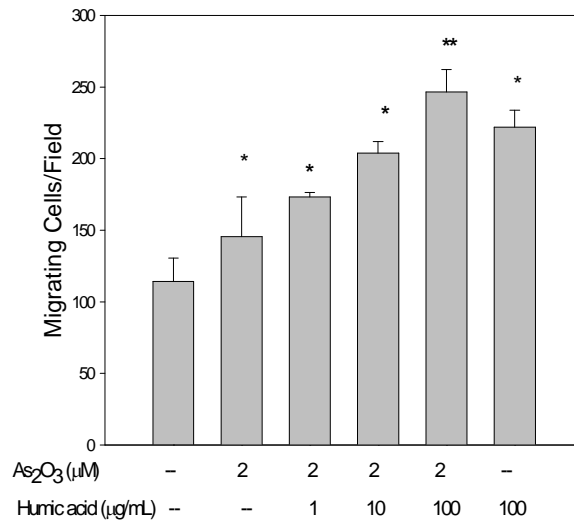


Fig.2 Effect of HA and arsenic oxide on the migration of JB6 C141 cells. JB6 C141 cells

were treated with various concentration of HA and 2 μM arsenic oxide for 48 h.

Then the cultures were wounded by scratching the monolayer of cells with a p200

ependorf pipet tip, and the mediums were again changed and incubated for another

48 h, then fixed in 2% paraformaldehyde in PBS, stained with 1% crystal violet and

photographed at 40 x magnification. The number of cells migrated into the wounded

area was counted.

Fig.3

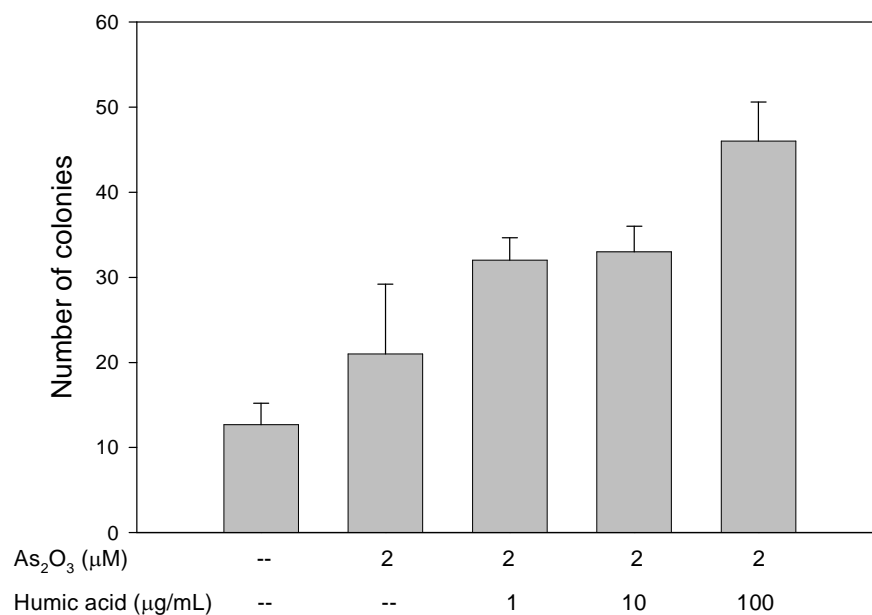


Fig.3 Foci formation assay. Foci formation assays of JB6 Cl41 cells were performed by the co-administration of various concentrations of HA and arsenic oxide for 48 h, and the cells were then washed with PBS and cultured in MEM supplemented with 5% serum for 6 weeks. The visual foci diameter above 3 mm with dense-stain were counted. The quantification is shown in the bar graph from at least three independent experiments. * $p < 0.05$, compared with control cells.

Fig.4

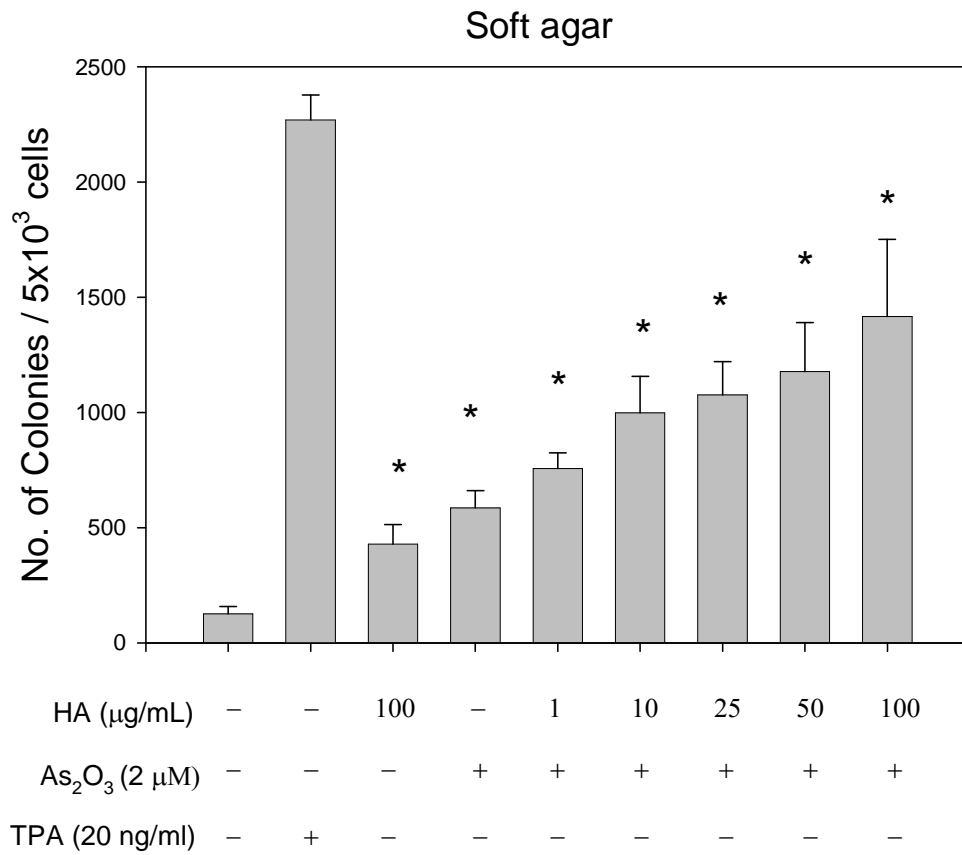


Fig.4 Anchorage-independent growth assay. The JB6 Cl41 cells were exposed to TPA (20 ng/ml) or different concentrations of HA and arsenic oxide for 48 h. The cells were harvested and seeded at a density of 5×10^3 into 0.33% soft agar over a 0.5% agar bottom layer. Cells were grown at 37 °C in 95% air plus 5% CO₂. Colonies with more than eight cells were counted. The results from three separate repair experiments are shown (mean \pm SD). * $p < 0.05$, compared to untreated control.

Fig.5

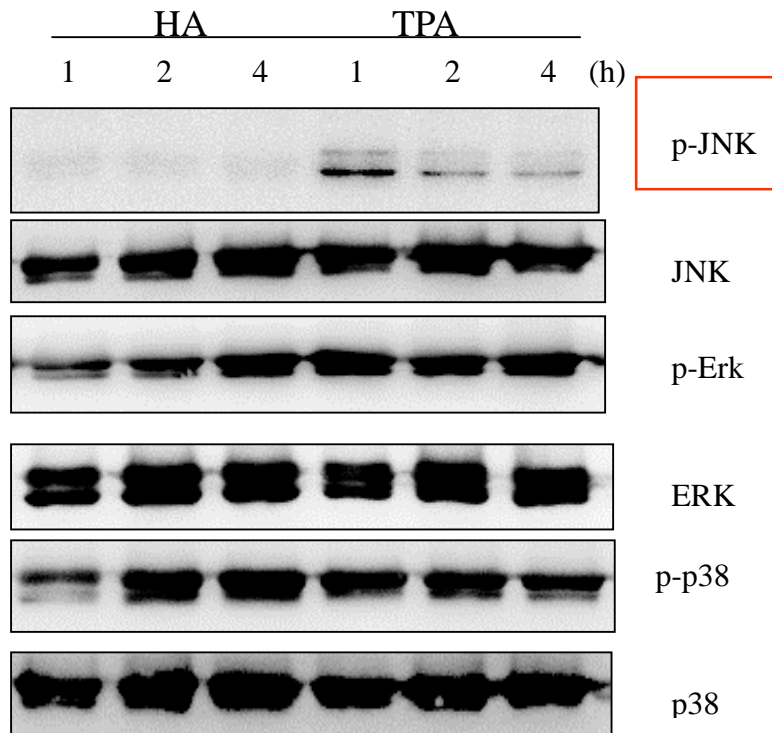


Fig.5 Effect of HA and TPA on MAPKs activation. Total cell lysates of JB6 Cl41 cells treated with HA or TPA for indicated times were extracted, and the phosphorylated JNK, ERK, and p38 MAPK (active form) were analyzed by SDS-PAGE and subsequently immunoblotted with antisera against MAPKs and its active form.

Fig.6

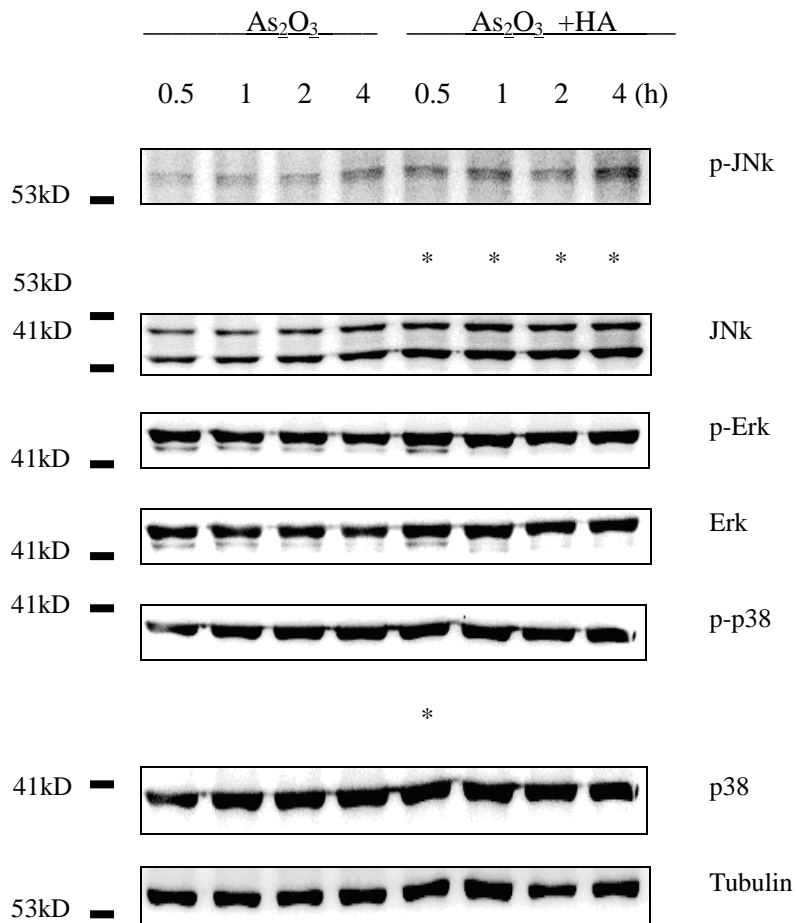


Fig.6 Effect of arsenic oxide and co-administrated with HA on MAPKs activation. Total cell lysates of JB6 Cl41 cells treated with arsenic or co-administration of arsenic oxide and HA for indicated times were extracted, and the phosphorylated JNK, ERK, and p38 MAPK (active form) were analyzed by SDS-PAGE and subsequently immunoblotted with antisera against MAPKs and its active form.

Fig.7

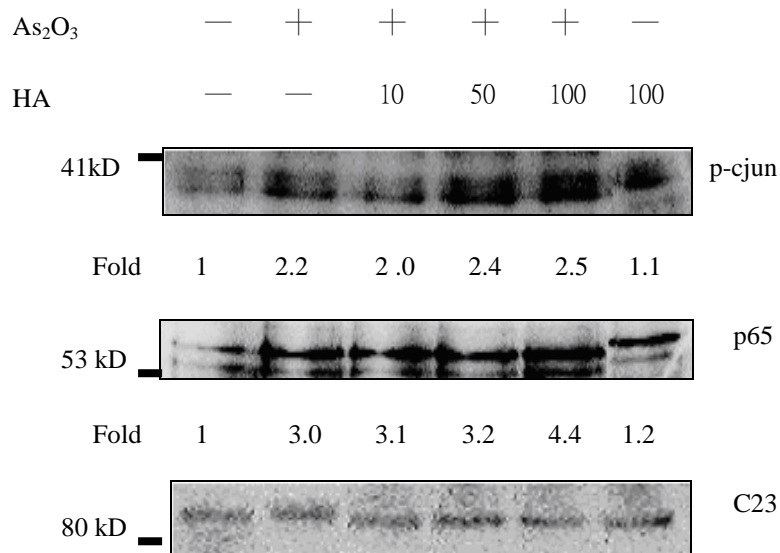


Fig.7. Effect of arsenic oxide and co- administrated with HA on MAPKs activation. Total nuclear lysates of JB6 Cl41 cells treated with arsenic or co-administration of arsenic oxide and HA for 4 h were extracted, and the p-cJun and p65 were analyzed by SDS-PAGE and subsequently immunoblotted with antisera against p-c-Jun, p65 and C23 (as internal control).

Fig.8

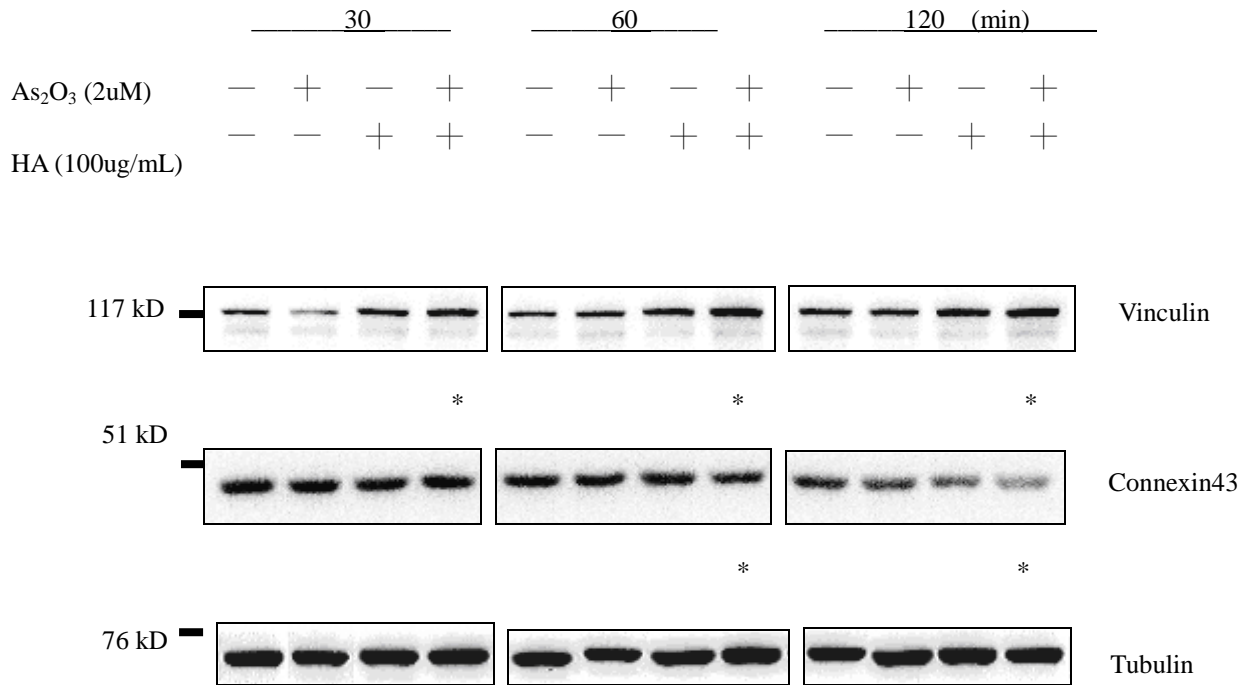
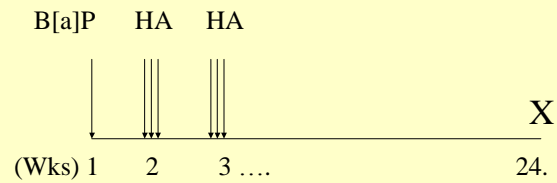


Fig.8 Effect of arsenic oxide, HA and co- administration on the expression of vinculin and connexin 43. Total cell lysates of JB6 Cl41 cells treated with arsenic, HA, and co- administration for 24 h were extracted, and the proteins were analyzed by SDS-PAGE and subsequently immunoblotted with antisera against vinculin and connexin 43.

Animal study



(B[a]P, benzo[a]pyrene)

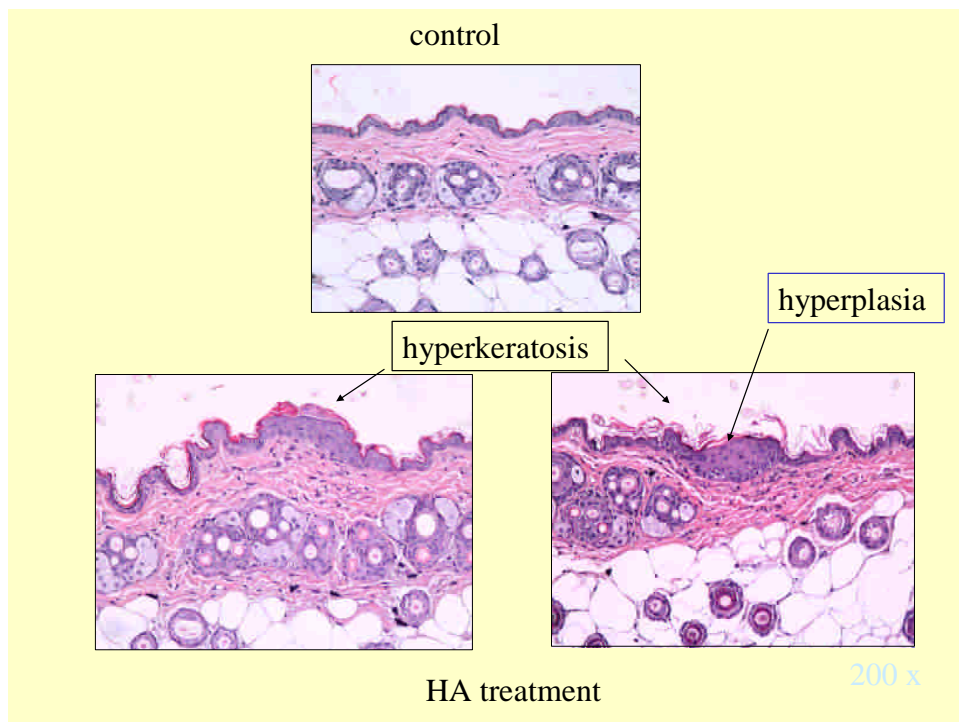


Fig.9 Effect of HA on epidermal hyperplasia in mice. Mice dorsal patches were treated with benzo[a]pyrene and HA as indicated. Skin biopsies were processed for hematoxylin and eosin staining. Section of the skin from a control animal treated with solvent (upper). Section of the skin from an animal treated with HA showing hyperplasia.

HA	—	+	—	—	0.05	0.25 (mg)
B[a]p	—	—	+	+	+	+
TPA	—	—	—	+	—	—

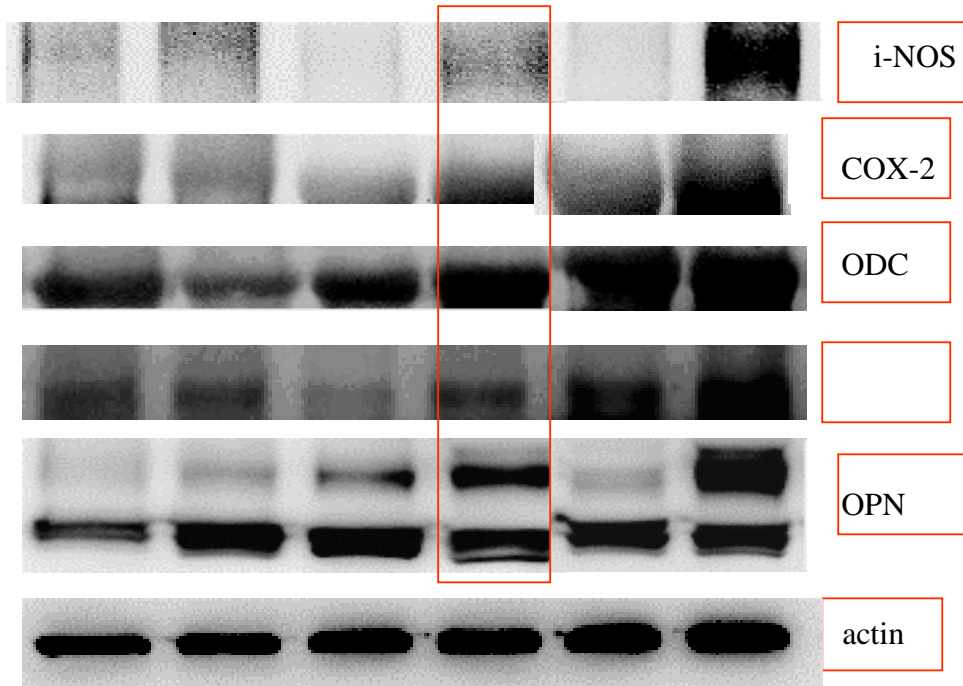


Fig.10 Effect of HA on the expression of epidermis COX-2, iNOS, ODC, PCNA, OPN in mice.

The mice were sacrificed and the epidermis was removed for immunoblotting analysis against anti-iNOS, anti-COX-2, anti-ODC, anti-PCNA, and anti-OPN. The data are presented from one mouse per group and each lane contained 80 μ g protein. Other rats in each group showed patterns of protein expression similar to the representation.