幽門桿菌小鼠感染模式之建立與應用研究

Development and application of *Helicobacter pylori*-infected *in vivo* Mice Model

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前言

幽門螺旋桿菌(*Helicobacter pylori*, HP) 在 1983 經 Marshall 披露為一個新發現的胃內致病菌後(A new gastric pathogen) 起，至今經過第一個十年的廣泛研究，引起了胃腸科學界相當大的震撼。目前已然了解此革蘭氏陰性桿菌(Gram negative bacilli)與胃十二指腸潰瘍(Gastroduodenal ulcers)、慢性胃炎(chronic gastritis),非潰瘍性消化不良(Non-ulcer dyspepsia)，甚至胃癌(Gastric cancer)及部份低惡性 B 細胞之黏膜層胃淋巴瘤(MALToma)有密切之相關[1-5]，而且藉著細菌的根除(Eradication)可改變臨床上其中部份疾病(特為潰瘍)治療之自然史，為世人注目重視[6-10]。

HP 本身為一同種菌類中具多元性菌株差異 (Bacterial diversity)之革蘭氏陰性桿菌(Gram negative bacteria) [9],依其菌類基因及分泌之蛋白分析可發現桿菌具有, *cagA, vacA gene, ice A, ure A, B, E gene, babA, iceA* 及 CagA, VacA proteins, adhesin, outer-membrane releasing proteins (ORM) 等等菌類基因及蛋白表現上之差異[11-26]。然而這些菌株上之差異，是否可造成不同疾病之致病機或者有地理性的特殊差異，目前世界上各個研究機構正如火如荼地進行深入之研究; 近年來微生物研究者亦致力運用臨床幽門桿菌培養(Clinical culture isolate)於小鼠(Mice)建立 *H. pylori* 感染之活體動物模式(*in vivo* animal model), 除可藉以間接地瞭解人類幽門桿菌感染臨床上目前未解答之各式各樣問題; 並且是提供基礎致病機轉(Pathogenesis),與多元性菌株差異是否引起不同臨床疾病自然史(Natural history)縱向評量(Longitudinal survey)之良好途徑;尤有甚者:動物模式更為發展免疫治療如治療性或預防用疫苗(Therapeutic or passive vaccines)之必備基礎。

本文以下將擇要介紹:

1. *H. pylori* 動物模式建立之沿革及重要性
2. *H. pylori* 動物模式建立之要點
3. 多元性菌株差異於 *H. pylori* 動物模式建立之影響
4. 動物模式於 *H. pylori* Transmission route 之評估應用
5. 應用 *H. pylori* 動物模式於 Ulcerogenesis 之探討
6. 應用 *H. pylori* 動物模式於 Gastric carcinogenesis 之探討

H. pylori 動物模式建立之沿革及重要性

由於1987-1988年間幽門桿菌培養成功之技巧大幅進步並且已較為廣泛運用,是以於 1990-1996 年間運用由臨床上培養而幽門桿菌,進一步執行動物模式之建立在各式各樣之動物上分別有成功之報告[27-34],其中運用小鼠之動物模式之建立更分別在 1997-1999 年間之 Science, Gastroenterology, Cancer research 等知名期刊中有多次建立與應用之報告, 台灣屬幽門桿菌感染相當高盛行之國家, 臨床上胃腸疾病幽門桿菌感染亦相當盛行,是故以國人臨床幽門桿菌培養建立之幽門桿菌感染動物模式及其應用,當有其重要及迫切性[35]。

H. pylori 小鼠模式建立之要點

動物模式之建立其中有很多細節值得注意,謹以下列要點與大家討論分析:

1. 種入菌類之新鮮度(Freshness of inoculates):

理論上每一隻 HP 均可以用來執行動物模式之建立；但在以同一隻 HP 建立模式時,需要以較年輕(Freshness)或稱之為離原有臨床培養株(Initial clinical isolates)較為接近之次培養株(Subculture isolates)為宜 ,一般而言, 若次培養株之再分離世代(Passage)超過 20 個世代,其動物模式建立成功著生感染之比率會大幅降低[36,37] 。是以動物模式之建立時,必須儘可能以 Freshness 高之世代執行種入。除此之外,種入菌類離開培養基(Culture medium),在種入小鼠前必須經過調整菌類濃度(Colony counts)及寄存液體培養介質(Broth aliquot)而後再轉運(Transport)至動物中心等待種入小鼠,除加速時間之掌握外(儘可能在不超過 30 分內完成種入), 在這期間種入菌類之

保存亦相當重要，一般必須將菌液保存在攝氏35渡度與5% Micro-aerophilic condition,如此動物模式建立成功之比率會較為穩定。

2. 種入菌類之濃度(Concentration of isolates)

在種入小鼠之菌類濃度當然是越高越容易成功,但是相對培養之成本與種入小鼠之數量與時序成效亦越高。一般而言，若以再分離 20 個世代之次培養株 0.3~0.5 ml x 10^9 CFU/ml 的幽門桿菌之桿狀體,連續三日執行種入，動物模式建立成功之比率會高達80-90%;但若將培養株種入小鼠之菌類濃度調低為 10^8 CFU/ml，則動物模式建立成功之比率會降低至 70 - 80%,依此類推 10^7 及 10^6 CFU/ml 動物模式建立比率分別僅存 50%及 30-40% [36-38,45]。

3. 小鼠經口種入菌類之頻次與技巧(Frequency and skill of oral garvage)

如何將菌類種入小鼠胃內是一個相當重要之步驟,一般之原則在於運用胃部餵食小管(Feeding)經口種入菌類(Oral garvage),此一步驟須要有良好之餵食技巧並且仔細瞭解到小鼠會厭喉管(Epiglottis larynx)及食道進口(Esophageal inlet)之解剖位置(Anatomy),進一步配合抓緊小鼠，固定頭頸與身體直線軸線(Head-body-axis),如此才能有效而且安全地將菌類種入小鼠胃內[35-38]。此一步驟不僅關係動物模式建立成功之比率,也是最容易造成小鼠致命之步驟,須要常常反覆勤練,再 Try and Error 中，找出來種入小鼠胃內之手感。也因為種入菌類具有造成小鼠致命之危險性,種入之頻次應求儘量減少,但在相同菌類濃度種入之前提下,減少種入之頻次明顯將會使動物模式建立成功之比率降低[45-48]。

4. 種入菌類小鼠之前置準備(Pretreatment protocol for mice) [35,39]

原則上我們須要讓小鼠之胃部在種入菌類小鼠之前空無一切,並儘量使其胃內之黏液減少,是以禁食 24-48 小時是常見之前置準備[35];另一方面運用去黏液藥物(e.g. pronase, peptidase, etc) 也是常見之前置準備[35,53];另外在使種入菌類能停留在小鼠之胃部有較長之時間，前置準備亦或會使用解痙注射(e.g. buscopan)或使用 Ketamine 腹膜腔注射以延長小鼠之胃排空時間(Gastric emptying time) [52,53]。

多元性菌株差異於 *H. pylori* 動物模式建立之影響

針對胃這一個原本對細菌是一塊處女地的器官,如何被幽門桿菌攻陷城池,以下經由細菌本身特性之分析做介紹,其中殊為值得建立之重要觀念,在於—細菌感染之建立(Infection)必須經由初期著生(Initial colonisation)及持續著生(Persistent colonisation),進而維持持續性感染(Maintenance of infection)三個步驟[36-41]。於各步驟中,細菌本身或胃內環境改變及宿主(Host)抗原之特異性可能扮演之正面或負面意義,茲列表於(附表一),其中一向被列為 high virulence factor of *H. pylori* 之 Vac A , Cag A protein 或 *cag A* / *pic A,B* pathogenic islands 竟與 Initial 或 Persistent colonisation 無關,而與維持持續性感染是否有關仍未確定,值得進一步分析研究。一般而言, Initial colonisation 與尿素胺(Urease),細菌鞭毛及相關之分泌附著因素(Adhesin)之關係最大[42-44]; 而 Persistent colonisation 取決於 Urease 與細菌之活動能力(Motility of *H. pylori*),以避免被感染宿主發炎或免疫細胞之清除[42-44]。在形成病理變化而改變胃內環境達到 Maintenance of infection 時 Adhesion 或 Antigen mimicry 之角色目前亦未明。一般要達成 Maintenance of infection 須各方〝可能〞因子(Possible factors)之共同配合(如表一), 有關在這方面應用動物感染模式施展免疫學之研究,將是未來揭開幽門桿菌與宿主之間感染之路(Interaction)的期待。

表一 幽門桿菌於初期及持續著生進而維持感染之有關因素

	Initial	***persistent***
A. Colonisation factors		
	urease ++	Motility ++
	Flagella ++	adhensin ?
	Adhensin - ?	antigen mimicry ?
	acid suppression - ??	inhibit PMN ??
	barrier disruption - ??	inhibit lymphocyte ??
B. Possible factors for maintenance of infection		
	- motility	- suppress immune cell
	- adhensin	- specific metabolic pathway
	- antigen mimicry	- survival inside cells

* This table is a summary from the reference [26-45].

臺灣地區屬幽門桿菌感染相當高盛行之國家, 臨床上胃腸疾病幽門桿菌感染 c*agA*-positive 感染相當盛行幾乎接近 100% [21],是以使用國人臨床幽門桿菌培養之幽門桿菌(Isolates)感染動物模式及其應用,當有其重要及迫切性。是以吾等由臨床收集之幽門桿菌培養(*H. pylori clinical isolate*),經幽門桿菌培養之菌株 *cagA* 基因差異分析後, 依藉國人臨床幽門桿菌培養所得之菌種目前可成功地於 BALB/c SPF (Specific pathogen free)小鼠建立幽門桿菌感染動物模式; 並發現:儘管 *cagA-positive* 菌種感染後之小鼠胃黏膜發炎反應明顯較 *cagA-negative* 菌種感染者明顯;但因 *cagA* 基因差異實施感染動物 BALB/c mice 模式之建立感染率並無差異(*cagA-positive vs. cagA-negative:* 91.4% *vs.* 89.2%, P > 0.05);由此可見,有關臺灣地區 *cagA-positive* 幽門桿菌菌種感染極高盛行率之原因,似乎可能不單由 *cagA* 基因差異來決定,值得進一步研究[35]。

動物模式於 *H. pylori* Transmission route 之評估應用

除了發現胃組織切片上有幽門桿菌存在，Marshall 與 Warren (1984) 亦發現胃組織上存有球形的幽門桿菌。之後，科學家們可以在 *in vitro* 環境下誘導球形體的形成，研究報告顯示當培養時間延長、營養狀況不好或在高氧含量以及抗生素刺激下，幽門桿菌會由原始的桿狀體轉型為球形體 [46,47]。球形體細菌也可能藉此形式傳播(Transmission)或躲避不適當(Avoidance)的生存條件[48]。此外，當培養液中的桿狀體比率佔全體的 30% 以下時，將無法再於培養基上次培養出幽門桿菌 [47]。至目前為止，在實驗室仍無有效的方法能將轉型為球形體的幽門桿菌回復成桿狀體。是以球形體幽門桿菌為死的或活的狀態，甚至於是否可能扮演傳染之重要圖途徑,至今在科學界仍有所爭議 [46-48]。

過去的研究方向則以不同層次的生化及細菌特性試圖來解答球形體的存活問題：由於從細菌的外觀來看，球形體和桿狀體同樣具有完整的細胞膜(Membrane)及鞭毛(Flagella) [47,49]；此外，動物模式方面，球形體無法感染 gnotobiotic piglet [50]，但是在 BALB/c 小鼠 (mice) 的胃中，球形體不僅能感染造成急性胃炎(Acute gastritis)，而且還會產生系統性的抗體反應(Immune response)，甚至還可以觀察到球形體在小鼠胃中回復成桿狀體 [51-53]。吾等在成功大學將培養於 37℃培養箱中桿狀體或球型體之菌液，以胃管將適當量的菌液直接餵食入 BALB/c 小鼠的胃中。餵食之後連續兩天，於小鼠的飲水中添加桿

狀體或球型體之菌液。以 10^8 CFU/ml 的幽門桿菌之桿狀體(Spiral form)及球形體(Coccoid form)菌液餵食BALB/c小鼠的附著比率分別為64.3% (9/14隻) 及66.7% (10/15 隻)，兩種型態的幽門桿菌皆具有類似的著生感染比率(Colonization rate)。若以 10^6 CFU/ml 的桿狀體菌液餵食 BALB/c 小鼠，結果發現其感染比率大幅降低，僅有 26.7% (4/15 隻) (P<0.05)。此結果顯示球形體幽門桿菌依然保有其感染能力。以組織切片化學染色來觀察，餵食桿狀體菌液的小鼠胃組織上會有桿狀體幽門桿菌的存在，有趣的是餵食球形體菌液的胃組織可發現桿狀體的幽門桿菌之存在，這結果顯示球形體在 BALB/c 小鼠的胃中可能有回復(Conversion)成桿狀體的能力，此發現確定 HP 細菌可藉球形體形式傳播(Transmission)或躲避不適當(Avoidance)的生存條件[53]。

應用 *H. pylori* 動物模式於 Ulcerogenesis pathway 之探討

臨床上感染 *H. pylori* 被視為引起胃十二指腸潰瘍(Gastroduodenal ulcers)之種要相關危險因子(Risk factor),但是其確定機轉仍需進一步研究:目前在 BALB/c 品種引發之 SCID (Severe combined immunodeficiency mice)動物感染模式上,已可以成功地引起潰瘍之產生[54,55],在吾等之實驗中可發現小鼠潰瘍缺口上除了幽門桿菌聚集以外,也明顯發現有大量之多核性白血球浸潤(Polymorphonuclear neutrophil infiltration)[55];另一方面在以醋酸(Acetic acid)先行引起胃十二指腸潰瘍之 BALB/c SPF mice 上,亦可發現幽門桿菌會抑制潰瘍缺口之血流進一步造成潰瘍缺口癒合之緩慢[56],預期應用動物感染模式以揭曉潰瘍形成奧秘之研究,將在不久之未來與世人見面。

應用 *H. pylori* 動物模式於 Gastric carcinogenesis 之研究

以往在小鼠投予以化學性致癌物質(Chemical carcinogen)，已然發現可引起小鼠產生胃癌(Adenocarcinoma) ;由於在 1996 年 Hirayama et al.發現在沙鼠(Mongolian gerbils)種入幽門桿菌後可成功地引起胃炎及潰瘍[56],甚至於產生胃黏膜萎縮(Gastric atrophy)及腸道上皮化生(Intestinal metaplasia)之癌症前兆病變(Precancerous lesions)[56,57];其後日本學者便更進一步地運用沙鼠幽門桿菌感染模式,執行建立胃癌之研究實驗,在 1997-2000 間有了相當重要之進展,此些進展可分為兩大方面:

1.運用化學性致癌物質配合幽門桿菌感染致癌之研究結果:

此一方面較常用化學性致癌物質為 MNU(*N*-methyl-*N*-nitroso-urea) 或 MNNG (*N*-methyl-*N*-nitro-*N*-nitosoguanidine);文獻上發現若配合幽門桿菌感染與 MNNG 或 MNU 可在沙鼠(Mongolian gerbils)種入幽門桿菌後,於 40-52 週時發現 25-60%致癌之危險比率[58-61] 。在 Shimizu et al.之研究中,更發現幽門桿菌會加速促成 MNNG 致癌之比率,而 MNNG 之濃度以 20ppm 而 100ppm 最易於與幽門桿菌產生加成致癌之比率[60];有趣的是在這些發表 MNNG 或 MNU 易於與幽門桿菌產生加成致癌之文獻中,作者們均未能在僅種入幽門桿菌之沙鼠獲得致癌,這個結果與以下第二部分之成果有異,值得住注意的是:可能是追蹤之時間有所不同。

2.僅種入幽門桿菌獲得致癌之沙鼠研究

在 1998 年 Watanabe et al.發現在沙鼠(Mongolian gerbils)種入日本本土(Domestic strains)胃潰瘍患者之幽門桿菌 62 weeks 後,有 37%之沙鼠可成功地引起胃癌[62];另一份重要之類似報告:Honda et al. 使用 ATCC43504 (*cagA*+ & *vacA*+)之標準菌種種入沙鼠,於 72 weeks 時,也可成功地引起分化良好之胃癌(Well differentiated adenocarcinoma)[63] 。這兩份研究完成了幽門桿菌是否致癌柯克假說(Koch's postulates) 之最好証明,也揭示幽門桿菌確定為 WHO 所言之 Type I carcinogen。著眼未來運用種入幽門桿菌致癌之沙鼠之模式將有助於胃癌致病機轉之釐清。

結語與展望

於小鼠(Mice) 建立 幽門桿菌感染之活體動物模式提供基礎致病機轉與多元性菌株差異是否引起不同臨床疾病自然史縱向評量之良好途徑;尤有甚者:動物模式更為發展免疫治療如治療性或預防用疫苗之必備基礎。然而目前致癌之模式僅只於沙鼠上可成功,未來進一步之相關研究仍有重要之突破空間。臺灣目前已成功建立本土之幽門桿菌感染動物模式,並在國科會與國家衛生院之支持下積極執行相關應用研究;然而動物模式之研究費時費力,且成本相當可觀,在此期許國內研究單位多方投入,也期望相關研究主管單位多所支持與鼓勵。

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