行政院國家科學委員會專題研究計劃成果報告
易脆 X 染色體症候群淋巴球對氧化性傷害高敏感機制之研究
Are the Lymphocytes from Fragile X Patients Supersensitive to Oxidative DNA Damages?

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一、摘要

易脆 X 染色體徵候群是最常見的遺傳性智能障礙疾病，造成原因，主要是由於 X 染色體上 *FMR1* 基因的第一個 exon 內 5'端不轉譯區的 CGG 三聯核　酸發生倍增性突變，且在起動區 (promoter region) 的 CpG island 常伴隨發生超甲基化，導致 *FMR1* 基因不表現。CGG 三聯核　酸的倍增突變以及 *FMR1* 基因的超甲基化，不但影響了 *FMR1* 基因的轉錄，同時也影響 DNA 的結構，而在低葉酸的培養環境表現出易脆 X 染色體 [FRAXA，Xq27.3]。

早期學者研究發現，在一些大腸癌患者其 DNA 核　酸同樣有不安定的現象，造成的原因是由於失誤配對修補 (mismatch repair)基因 *hMSH1*, *hMSH2* 的突變；至於易脆 X 染色體徵候群患者細胞是否因為核酸修補的異常，而導致 CGG 三聯核　酸的倍增突變是值得深入研究的。

本研究取三個完全突變型之易脆 X 染色體徵候群 (TN272、TN411 及 CT441)和兩個正常人 (N2 及 N6) 的淋巴球細胞株。三個易脆 X 染色體症候群淋巴球細胞株 TN272、TN411 和 CT441 經細胞遺傳學方法檢測易脆 X 染色體的發生頻率；利用南方點墨法檢測 FMR1 基因 exon 1 不轉譯區域 CGG 三聯核　酸倍增的長度，並以西方點墨法進行 FMR 1 基因蛋白產物，FMRP 的分析，結果都證實此三株源自易脆 X 染色體症候群的細胞保留有高頻率易脆 X 染色體表現、CGG 三聯核　酸明顯倍增和 FMRP 低表現量的各項特性。利用造成不同型態 DNA 損傷、並藉由不同修補途徑進行修補的各種 DNA 傷害劑，包括氧化傷害劑 hydrogen peroxide 及 bleomycin；烷化劑 ethyl methanesulfonate；紫外線類似劑 4-nitroquinoline N-oxide；核酸交聯劑 mitomycin C 、核酸拓樸酵素 II 抑制劑 etoposide 及葉酸代謝抑制劑 methotrexate 來處理細胞，並以簡便且敏感度高的單細胞電泳分析，來了解 DNA 傷害程度及修補能力在易脆 X 染色體徵候群患者細胞株，三聯核　酸發生倍增突變所扮演的角色。另外本研究也對正常及易脆 X 染色體症候群，在低葉酸環境下，對自發性及上述核酸傷害劑引發 DNA 傷害及修補能力的影響進行分析，並以尿嘧啶糖基水解酵素 (uracil DNA glycosylase) 合併單細胞電泳的方式，分析尿嘧啶在低葉酸環境，在正常及易脆 X 染色體徵候群細胞株失誤嵌入 DNA 情形的比較。

本研究發現：(1). H_2O_2 在三株源自易脆 X 染色體的淋巴球細胞株 TN272、TN411、CT441 及 N6 相對於 N2 細胞株，造成較高的 DNA 鏈斷傷害且修補的能力也較低；(2). 低葉酸環境在 TN272 細胞株對 H_2O_2 引起 DNA 鏈斷傷害後修補，相對於 N2 細胞株，有較高的抑制作用；(3). 三株源自易脆 X 染色體的淋巴球細胞株 TN272、TN411 及 CT441 有較高的自發性 DNA 鏈斷傷害；(4) 易脆 X 染色體症候群細胞株及正常細胞株對 bleomycin 、4-nitroquinoline N-oxide 、ethyl methanesulfonate 及 etoposide 所造成的 DNA 傷害和修補並無明顯差異。本研究也發現幾個意外的結果：(1).

Mitomycin C 所造成的 DNA 交聯經 48 小時，仍無明顯修補；(2). 低葉酸環境培養 10 天，無法明顯測到在 N2 及 TN272 細胞，尿嘧啶失誤嵌入 DNA 的程度。上述研究結果顯示：易脆 X 染色體症候群 CGG 三聯核 酸發生倍增突變，經由細胞內 DNA 修補途徑缺陷所造成的可能性並不高。

關鍵詞：易脆 X 染色體症候群、淋巴母細胞、核酸修補

Abstract

Fragile X syndrome is the most frequents cause of heritable mental retardation. Most patients have a mutation in the 5'-untranslated region of FMR1 gene, consisting of the amplification of a polymorphic (CGG)n repeat sequence, and cytogenetically express the folate-sensitive fragile site FRAXA in Xq27.3. The molecular mechanism of amplification of CGG trinucleotide in fragile X syndrome is still unknown.

In the present study, cultivated human lymphoblastoid cell lines that derived from fragile X syndrome patients and normal individuals were damaged by H_2O_2, bleomycin (BLM), ethyl methansulfonate (EMS), 4-nitroquinoline-N-oxide (4-NQO), etoposide, and mitomycin C, the initial DNA damage and removal were detected by comet assay, a sensitive method for detecting strand breaks at the level of individual cells.

Among the cell lines of N2, n6, TN272, TN411, and CT441, the level of initial DNA damage and its removal by H_2O_2, BLM, EMS, 4-NQO, etoposide, and mitomycin C are similar. In addition, the effect of folate depletion on uracil misincorporation in lymphoblatoid cells was investigated by a modified "comet assay" using the bacterial DNA repair uracil DNA glycosylase. At the folate depletion condition, the increase level of uracil in lymphoblastoid cell lines both derived from fragile X syndrome patients and normal individuals are all untectable. These results suggest that CGG repeat instability in fragile X syndrome is not from endogenous or inducible DNA repair pathway deficiency in somatic cells.

Keywords: Fragile X syndrome, Lymphoblatoid cells, DNA repair,

二、緣由與目的

Expansion of repetitive tracts of trinucleotide repeats (TRS) in the human genome is associated with about 12 diseases including Fragile X syndrome and myotonic dystrophy (Ashley & Warren, 1995; Richards & Sutherland, 1995). All but one of these diseases, Friedreich's ataxia (Campuzano et al., 1996), results from expansion of a CCG/CGG or a CTG/CAG tract. Fragile X syndrome, a form of X-linked mental retardation is caused by unstable expansion of a CGG repeat in the 5' untranslated region of the FMR1 gene (Fu et al., 1991). Under normal circumstances, this CGG repeat is polymorphic, with individuals possessing 6-54 copies of the repeat (Eichler et al., 1994). The number of repeats usually is inherited without change, from parent to child. In families with fragile X syndrome, however, the repeat has a propensity to expand in size, in each generation. This phenomenon is also known as "anticipation".

The mechanism by which DNA repair defect increases the trinucleotide repeats instability of fragile X syndrome are unclear. To date, the only tractable assays for TRS instability involved physical monitoring of tract length alternations. In *Escherichia coli* and in yeast system, long trinucleotide repeats are unstable (White et al., 1999; Freudenreich et al., 1997; Miret et al.,

1997). Unlike affects human kindreds, large contractions are the overwhelming majority of products in these assays systems; expansions are rarely observed. Instability is strongly dependent on trinucleotide repeats in a particular orientation relative to an origin on DNA replication (Hirst & White, 1998). Little evidence is available for these systems regarding the roles of specific cellular factors. Mutations in the *Escherichia coli* mutS, mutL, and mutH mismatch repair pathway significantly stabilize the repeats, suggest that mismatch repair plays an active role in the mutational process for trinucleotide repeats (Jaworski et al., 1995). Strains containing disruptions of the mismatch repair gene MSH2, MSH3, or PMS1 or the recombination gene RAD52 showed little or no difference in rates of instability or distributions of products, suggest that neither mismatch repair nor recombination plays an important role in large contraction of CAG trinucleotide repeats in yeast (Strand et al., 1993). In mammalian cell lines deficient in mismatch repair, the trinucleotide repeat lengths at the fragile X and myotonic dystrophy loci were within normal ranges, suggest that mutations in hMLH or hMSH2 do not contribute to instability of the CGG or CTG tracts in these genes (Boyer et al., 1995). Cell lines deficient in another human mismatch repair gene, hPMS2, do exhibit instability of a CTT microsatellite sequence, but only small numbers of repeats appear to be altered in a given event. Thus there is no consensus on the effects of DNA repair on trinucleotide repeat stability.

Two basic models have been employed to explain trinucleotide repeat instability the first associated with recombination and the second associates it with the replication process. Recombination models explain the increase in the number of repeats as unequal crossover or gene conversion between homologous chromosomes or sister chromatids. The alternative slippage-during-replication model suggests that during replication the template and the new strand dissociate from each other. One of the DNA strands creates a new structure, for example, a hairpin, which results in contraction or expansion in the next generation, depending on which strand created the hairpin. In fact, the recent results show that trinucleotide repeat and palindromic sequence is form hairpin or cruciform structures (Moore et al., 1999). However, the resolving mechanism of hairpin or cruciform structures are still unclear, while structure-specific nuclease, introduction of DNA single strand or double strand breaks, and recombination system may be important as a general mechanism for the maintenance of repeat stability in mammalian cells.

Furthermore, our previous result show that the lymphocytes of fragile X patients are hypersensitive to BLM-induced chromosome aberrations (Li et al., 1992) suggest that DNA repair mechanism may be deficient in the lymphocytes of fragile X patients. To further elucidate whether a specific DNA repair pathway defect causes TRS of fragile X syndrome, we studies various DNA damaging agents induced DNA damages and repair capacities in Epstein barr virus (EBV)-transformed normal and fragile X syndrome lymphoblastoid cell lines, using single cell gel electrophoresis (SCGE or comet assay).

三、結果與討論

The lymphoblastoid cell lines used in this study was established by EB virus-transforming of lymphocytes that derived from normal individuals and fragile X syndrome patients. The characteristics of these lymphoblastoid cell lines were summarized in Table 1.

The induction percentages of fragile site, Xq27.3 were 42, 30, and 35% of three fragile X syndrome TNT272, TN411, and CT441 lymphoblastoid cell lines, respectively. However, the induction percentage of fragile site, Xq27.3 of two normal lymphoblastoid cell lines (N2 and N6) were not observed any metaphase cell with fragile site, Xq27.3 from 100 metaphase cells checking. The length of CGG trinucleotide repeat at 5' untranslated region of FMR1 gene of three lymphoblastoid cell lines (TN272, TN411, and CT441) were 2.4, 2.0, and 3.0 kb, respectively, longer than two normal lymphoblastoid cell lines (N2 and N6). The expression of FRM1 gene encoded protein, FMRP in normal lymphoblastoid cell line. N2 was significantly higher than two fragile X syndrome lymphoblastoid cell lines (TN272 and TN411), while a lymphobalstoid cell line, CT441 derived from a female fragile X patient also had a lower extent expression. This result indicated that CT441 cell line was a mosaic form of FMR1 gene expression. Taken together, these dada indicated that EBV transformation method conserved the basic characteristics of fragile X syndrome, such as the rare fragile site, Xq27.3 induction in folate-depleted conditional medium, no or low FMRP expression, and CGG trinucleotide repeat expansion.

For quantitative evaluation by Comet parameters, preliminary experiments with the genotoxic substances were performed to determine the suitable dose range and repair hours to be tested in N2 cells. In N2 cells, the dose for inducing $Max_{1/2}$ of DNA damage score, 200 for BLM, H_2O_2, EMS, 4-NQO, and MMC were 13.7 μg/ml, 25.0 μM, 136.7 μM, and 26.2 μM, respectively (Fig. 5A~E). The DNA repair half time ($t_{1/2}$) for BLM-, H_2O_2-, EMS and 4-NQO-induced DNA strand breakage were 1.5, <1, >4, and <1 hours respectively (Fig. 5H~J).

The extent of strand breakage, analyzed by Comet assay, in the lymphoblastoid cell lines, N2, N6, TN272, TN411, and CT441 following treatment with a single time and dose exposure of BLM-, H_2O_2-, EMS-, and 4-NQO was shown in Table 2. These data show that the DNA strand breakage was significantly increased by BLM, H_2O_2, EMS and 4- NQO treatments. There are slightly higher DNA damage extent and lower repair rate of H_2O_2-induced strand breakage in all three lymphoblastoid cell lines derived from fragile X syndrome and one cell lines derived from normal individual when compared with N2 normal cell line. This phenomenon is correlated with its slower repair rate of H_2O_2-induced strand breaks.

MMC causes formation of DNA cross-links that prevent DNA from migration in the Comet assay (Olive et al., 1995). Therefore, the extent of DNA damage by this drug is measured by observing the degree to which hydrogen peroxide-induced DNA strand breaks are "masked" by the cross-links. Figure 2E show the effect of mitomycin C-treatment (2 hr) on H_2O_2-induced DNA migration. When MMC-treated N2 cells were exposure with H_2O_2 (30 μM) at the end of MMC treatment and directly analyzed in the comet assay, a clear reducing effect on DNA migration was seen. While exposure with H_2O_2 alone leads to a strongly increased DNA damage score (300 compared to 20 in controls), mitomycin (10 to 100 μg/ml) caused a concentration-related decrease in H_2O_2-induced DNA migration. At 50 μg/ml MMC, H_2O_2-induced DNA migration was inhibited 52, 36, 26, 49, and 50% in N2, N6, TN272, TN411, and CT441 lymphoblastoid cells, respectively (Table 4). These data indicated that the reducing effect of mitomycin C on H_2O_2-induced DNA migration was 20% less in N6 and CT272 than N6, CT411 and TN441

lymphoblastoid cells, however this phenomenon was not consistent with fragile X syndrome.

Overall DNA strand breaks and DNA double strand breaks can be detected in individual cell exposed to etoposide when examined using alkaline an neutral comet assay, respectively (Table 3). Etoposide 100 μg/ml-induced DNA overall DNA damage and double strand breaks and the repair capacity during 1 hr were similar among N2, N6, TN272, TN411, and CT441 lymphoblastoid cell lines (Table 3). These data shown that etoposide-induced DNA double strand breaks was 3-fold less than etoposide-induced overall DNA strand breaks. On the contrast, the repair capacity during 1 hr of etoposide-induced DNA double strand breaks were 20% more than the repair capacity of etoposide-induced DNA overall strand breaks in all the cell lines. These dada indicated that etoposide-induced DNA double strand breaks was quickly repair than etoposide-induced overall DNA strand breaks.

In the present work, we have shown that the extent of DNA damage and ability of DNA damage repair induced by oxidative stressed agents, H_2O_2 and BLM; alkylating agent, EMS, UV-mimic agent, 4-NQO; DNA cross-linking agent, MMC; DNA DSB damaging agents are similar between normal and fragile X syndrome lymphoblastoid cell lines. These DNA damage agents cause different types of DNA damage and the DNA damages were repaired by different repair pathways (Table 5). To our knowledge, this is the first time that DNA repair defects has been proven to be less associated with trinucleotide repeat expansion in somatic human lymphobastoid cell system by comet assay. We propose that a meiotic-specific hairpin structure repair pathway deficiency is a very possible mechanism for trinucleotide repeat expansion.

三、計劃成果自評

This project was the first time to evaluate the correlation between somatic cell overall ability for base excision repair, nucleotide excision repair, and DNA single- and double-stranded breaks repair both in the lymphobastoid cells that derived from fragile X patients and normal individuals. The results was valuable for understanding the possibility of DNA repair defects on molecular mechanism of trinucleotide repeat expansion. The results of these projects are just in preparation for publishing.

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