ศึกษาอะพอพโทสิสในเซลล์เม็ดเลือดขาวจากผู้ป่วยมะเร็งปากมดลูกที่ได้รับรังสีรักษา

นางสาว พิสุทธิ์ ปามุทา

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STUDY THE APOPTOSIS IN LYMPHOCYTES FROM CERVICAL CANCER PATIENTS RECEIVING RADIATION THERAPY

Miss Pisut Pamutha

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พิสุทธิ์ ปามุทา : ศึกษาอะพอพโทสิสในเซลล์เม็ดเลือดขาวจากผู้ป่วยมะเร็งปากมดลูกที่ได้รับรังสีรักษา (Study the Apoptosis in Lymphocytes from Cervical Cancer Patients Receiving Radiation Therapy) อ. ที่ปรึกษา : รศ.พญ. ธาดา สืบหลินวงศ์, อ. ที่ปรึกษาร่วม : ผศ.นพ. ประยุทธ โรจน์พรประดิษฐ์ ; 96 หน้า ISBN 974-13-0493-5

อะพอพโทสิส (Apoptosis) หรือกระบวนการตายของเซลล์ ซึ่งสามารถชักนำให้เกิดขึ้นในเม็ดเลือดขาวจากกระแสเลือดได้โดยการฉายรังสีขนาดต่ำๆ การศึกษาในครั้งนี้เป็นแบบ cross sectional study โดยศึกษา percent apoptotic activity ในเซลล์เม็ดเลือดขาวที่ได้จากกระแสเลือดของผู้ป่วยมะเร็งปากมดลูกที่ได้รับรังสีรักษา ณ โรงพยาบาลจุฬาลงกรณ์ โดยทำการเก็บตัวอย่างจากผู้ป่วยมะเร็งปากมดลูกระยะละ 5 คน ในช่วงก่อนรังสีรักษาและหลังเสร็จสิ้นการฉายรังสีแล้วที่ระยะ 1 เดือน, 3 เดือน, 1 ปี และ 5 ปี กลุ่มควบคุมได้จากการเก็บตัวอย่างเลือดของผู้มาบริจาคโลหิตหญิงที่มีอายุใกล้เคียงกับผู้ป่วยมะเร็งปากมดลูกก่อนได้รับรังสีรักษาในแต่ละระยะ รวม 15 คน จากศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทย การศึกษากระทำโดยแยกเม็ดเลือดขาวออกและนำไปฉายรังสีที่ระดับ 0.5 Gy จากแหล่งรังสี Cobalt เพื่อชักนำให้เกิดอะพอพโทสิส ตรวจสอบเซลล์ที่เกิดอะพอพโทสิสที่เวลา 24 และ 48 ชั่วโมงหลังจากชักนำด้วยการฉายรังสีด้วยวิธี *in situ* terminal deoxynucleotidyl transferase (TdT assay) และตรวจนับเซลล์โดยใช้กล้อง Fluorescence microscope คำนวณหาค่า apoptotic index และ percent apoptotic activity ผลการศึกษาพบว่า percent apoptotic activity ในกลุ่มควบคุมมีค่าสูงกว่าผู้ป่วยมะเร็งปากมดลูกก่อนได้รับรังสีรักษา (p=0.001) แต่ไม่แตกต่างจากกลุ่มผู้ป่วยที่ได้รับรังสีรักษาครบแล้ว 5 ปี (p=0.079) การเปรียบเทียบ percent apoptotic activity ในกลุ่มก่อนการรักษากับหลังเสร็จสิ้นการรักษาที่ระยะ 1 เดือน, 3 เดือน, 1 ปี และ 5 ปี พบว่า percent apoptotic activity มีค่าเพิ่มขึ้นหลังการรักษาแต่ไม่มีนัยสำคัญทางสถิติ ค่า percent apoptotic activity ในผู้ป่วยมะเร็งปากมดลูกระยะต้นสูงกว่าในระยะปลาย (p=0.024) อาจจะสรุปว่า percent apoptotic activity น่าจะสามารถใช้เป็นดัชนีบ่งชี้ถึงการกลับมาเป็นอีกของโรคมะเร็งในการติดตามผลระยะยาวได้

หลักสูตร วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....
สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา.....
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PISUT PAMUTHA : STUDY THE APOPTOSIS IN LYMPHOCYTES FROM CERVICAL CANCER PATIENTS RECEIVING RADIATION THERAPY. THESIS ADVISOR : ASSOC. PROF. TADA SUEBLINVONG, M.D. , THESIS COADVISOR : ASSIST. PROF. PRAYUTH ROJPORNPRADIT, M.D. 96 pp. ISBN 974-13-0493-5

Apoptosis or programmed cell death in the peripheral leukocytes can be induced by low dose irradiation. In this cross section designed study, we studied the percent apoptotic activity found in lymphocytes obtained from peripheral blood of the cervical cancer patients who had received radiation therapy at King Chulalongkorn Memorial Hospital. A group of five patients in each stage of cervical cancer and at the designed posttreatment intervals were included. Blood samples were taken from individual patient of each group before the radiation therapy and after completion of radiation therapy at 1 month, 3 months, 1 year, and 5 years. The control samples were collected from healthy female blood donors at National Blood Bank, The Thai Red Cross Society, with age matched to each group of pretreated cervical cancer patients. The lymphocytes were separated and irradiated with 0.5 Gy Co radiation to induce apoptosis. The apoptotic cells at 24 and 48 hours after radiation induction were detected by *in situ* terminal deoxynucleotidyl transferase (TdT assay) and counted under fluorescence microscope. Both the apoptotic index and percent apoptotic activity were calculated. The results showed that the percent apoptotic activity in the control groups was higher than the pretreated cervical cancer patients ($p=0.001$), but it was not differed from the 5 years posttreated groups ($p=0.079$). Comparison of the percent apoptotic activity in pretreated groups to posttreated groups at 1 month, 3 months, 1 year, and 5 years resulted in an increment of the percent apoptotic activity in the posttreated groups without any statistical significance. Higher percent apoptotic activity was observed in patients with early stage cervical cancer than late stage ($p=0.024$). In conclusion, it may be possible to apply the percent apoptotic activity as a parameter to detect any recurrent cancer in long term follow up.

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Academic year 2000

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Advisor's signature……
Co-advisor's signature……..

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List of abbreviations

DNA	Deoxyribonucleic acid
rpm	Revolution per minute
g	Gram
ml	Millititre
µl	Microlitre
M	Molar
°C	Degree Celsius
Gy	Gray
pH	The negative logarithm of the concentration of hydrogen ions
FITC	Fluorescein isothiocyanate-conjugated
FIGO	International Federation of Gynecology and Obstetrics
ASR	Age standardized rate

CHAPTER I

INTRODUCTION

1. Background and Rationale

The report on cancer in Thailand vol. II published by the Ministry of Public Health in 1999 showed that cancer of the cervix is the most common cancer found in Thai women with an estimate of 5,462 new cases in 1993 (Figure 1). The highest incidence is located in Chiang Mai followed by Lampang, Bangkok, Khon Kaen and Songkhla .[1] A King Chulalongkorn Memorial Hospital, the incidence of cervical cancer was the first common cancer (14.81 %, 375/2,532) out of the ten leading cancer for in-patients and the first (25.70 % ,375/1,459) of the ten leading cancer of female in 1998.[2]

In general, rates of cervical cancer are higher among the economically developing societies, and lower in the developed world. The age-specific incidence showed that there is an early increase before aged 20, steep rise to about aged 45-50 years old and followed by a plateau then declined (Figure 2).[1]

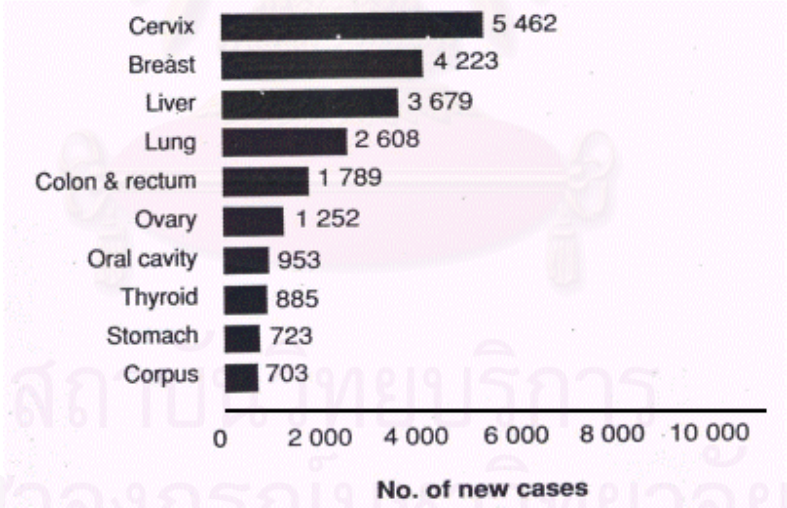


Figure 1 Leading cancer of female in Thailand in 1993 [1]

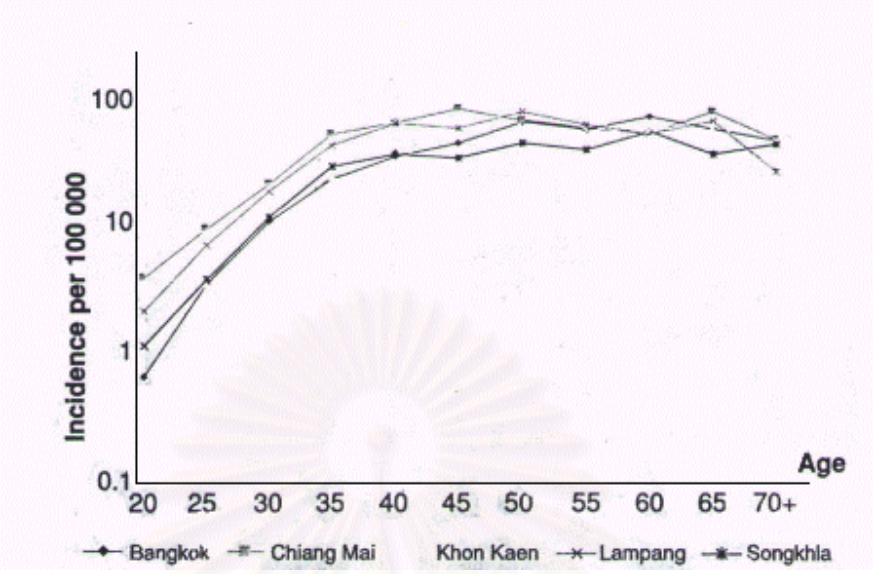


Figure 2 The age - specific incidence of cervical cancer.[1]

The most common risk factors in cervical cancer is the sexually transmitted human papillomaviruses (HPVs). Women infected with HPV-16 and HPV-18 exhibit a 60-fold greater risk of developing cervical cancer than uninfected women; both types have been identified in approximately 84 % of cervical cancer. However, the number of sexual partners are risk factor of cervical cancer that are now thought to be mediated by HPV. Cigarette smoking and taking steroid oral contraceptives has been shown to associate with an increasing risk of cervix cancer in many studies. In addition, diets high in vegetables, fruits, carotenoids, vitamin C and E found in foods of plant origin have possibly protective effect against cancer. Survival rates for cervical cancer are generally high, most of the early cases have high five year survival after initial diagnosis. The survival rates are considerably better for cancer cases diagnosed at early stage.[1]

Identification of staging is important before institution of therapy. The initial staging system proposed in 1929 by a subcommittee of the League of Nations was later revised in 1937 and 1950. This

function was taken over by FIGO in collaboration with the World Health Organization and the International Union Against Cancer. The staging recommendations were last revised in 1995, as shown in Table 1.[3]

Table 1 FIGO staging of carcinoma of the cervix uteri, 1995.[3,4]

Stage I	Carcinoma is strictly confined to the cervix (extension to the corpus should be disregarded)
	IA Invasive cancer identified only microscopically
	IA1 Measured invasion of stroma no greater than 3 mm in depth and no wider than 7 mm
	IA2 Measured invasion of stroma greater than 3 mm and no greater than 5 mm and no wider than 7 mm
	IB clinical lesion confined to the cervix or preclinical lesion greater than IA
	IB1 Clinical lesion no greater than 4 cm in size
	IB2 Clinical lesion greater than 4 cm in size
Stage II	carcinoma extends beyond the cervix but has not extended on to the pelvic wall; the carcinoma involves the vagina but not as far as the lower third
	IIA No obvious parametrial involvement
	IIB Obvious parametrial involvement
Stage III	Carcinoma has extended to the pelvic wall, on rectal examination, there is no cancer-free space between the tumor and pelvic wall; the tumor involves the lower third of the vagina, all cases with a hydronephrosis or non functioning kidney should be included unless they are known to be due to other cause
	IIIA Involvement of the lower third of vagina
	IIIB Extension onto the pelvic wall or hydronephrosis or non functioning kidney

Stage IV	Carcinoma has extended beyond the true pelvis or has clinically involved the mucosa of the bladder or rectum
	IVA Spread of the growth to adjacent organs
	IVB Spread to distant organs

The incidence rate of cervical cancer in Thailand when compares with other countries is that cancer of the cervix is the seventh most common cancer in the world. In 1990, an estimated 371,100 new cases were diagnosed world-wide, accounting for 4.6 % of all new cancers. Cervical cancer is the third most common cancer of women. The highest incidence rates are seen in part of Sub-Saharan Africa, and Latin America. Developing countries account for 80 % of the total cases. In Thailand, the incidence rate of cervical cancer is intermediate. The lowest rates are found in North America, Western Europe and a few nations in the Eastern Mediterranean. The incidence and mortality rates of the cervix cancer in 15 selected countries are shown in figure 3. [1]

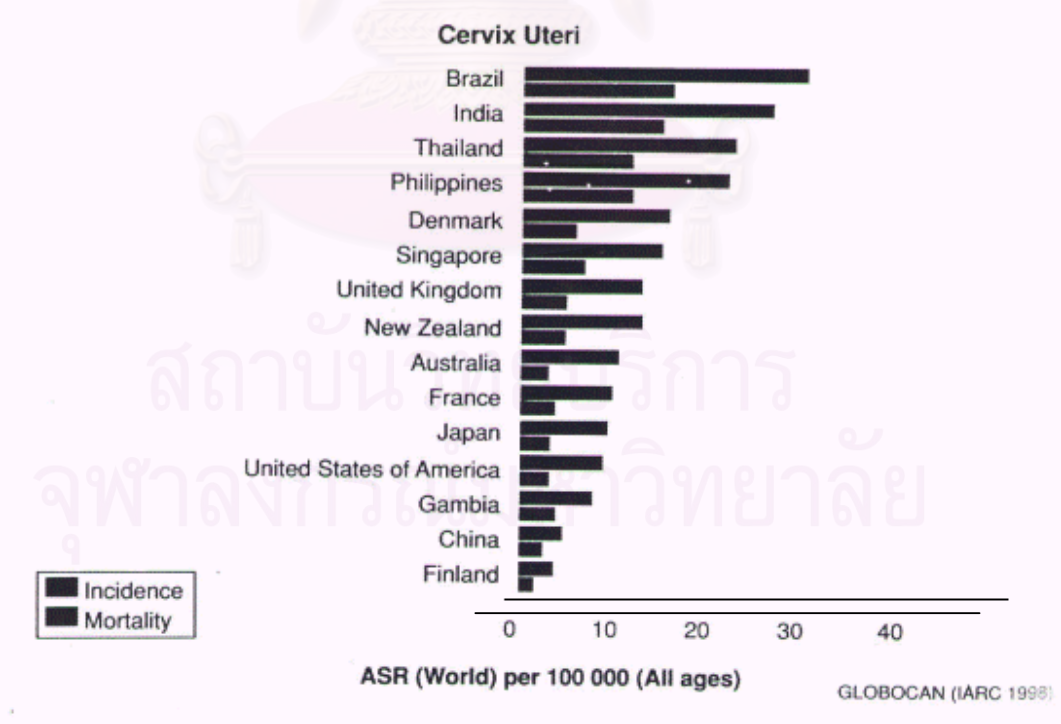


Figure 3 Incidence and mortality rates of cervical cancer.[1]

Each year, among women in the developing world, cervical cancer is the second most common cancer and the most frequent cause of cancer death estimated around 190,000 women worldwide.[1]

Currently, treatment of cervical cancer consists of surgery, chemotherapy, and radiotherapy. The treatment regimen for each patient is based on staging, invasiveness, and the response to treatment. The regimen may be a combination between irradiation and surgery, irradiation and chemotherapy or chemotherapy and surgery etc. Radiation is the most important therapy in cervical cancer. It is used in every stage of cervical cancer and the efficacy depends mainly on the total radiation dose which is limited by the tolerance of normal tissues surrounding the tumor. Normal tissue tolerance is widely varied among patients and hence the reactions to the same curative dose.[3,4]

Tumourigenesis is possibly started with stimulations from chemical agents, heat, and radiation etc. causing changes of both biological and cytoplasmic material in the cell leading to the generation of free radicals and activated caspases enzyme inducing DNA damage. When DNA damage occurred, there are 3 possible fates: 1) DNA damage is repaired back to normal via DNA repairing mechanism, 2) when the DNA damage seems irreparable, the affected cell will engage in a suicidal process (apoptosis). Apoptosis is a protective mechanism against tumourigenesis since death cell cannot turn into the cancer cell, 3) If the cell does not die nor repairing itself to normal then it or its progeny may live long to accumulate mutation that turn them to become the cancer cells (Figure 4).[4]

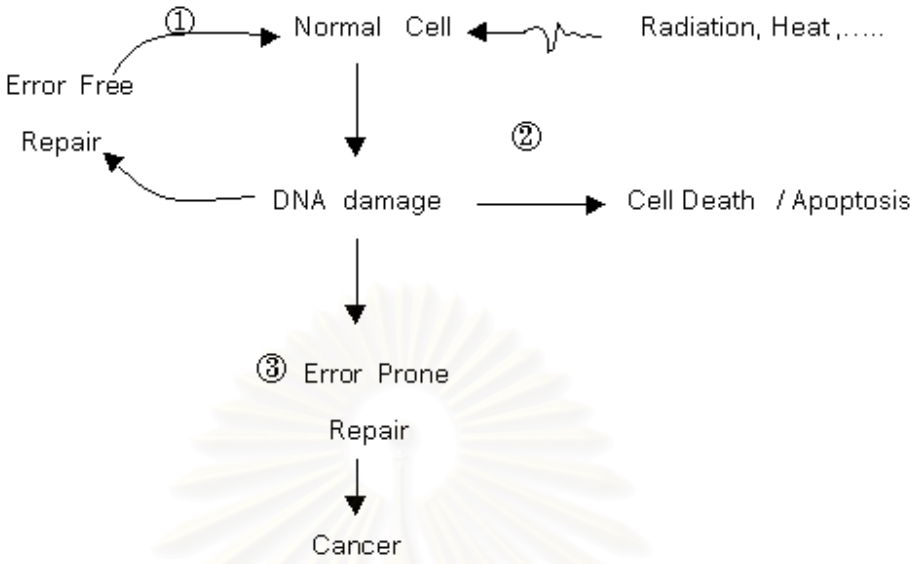


Figure 4 The fates of cell with damaged DNA.[4]

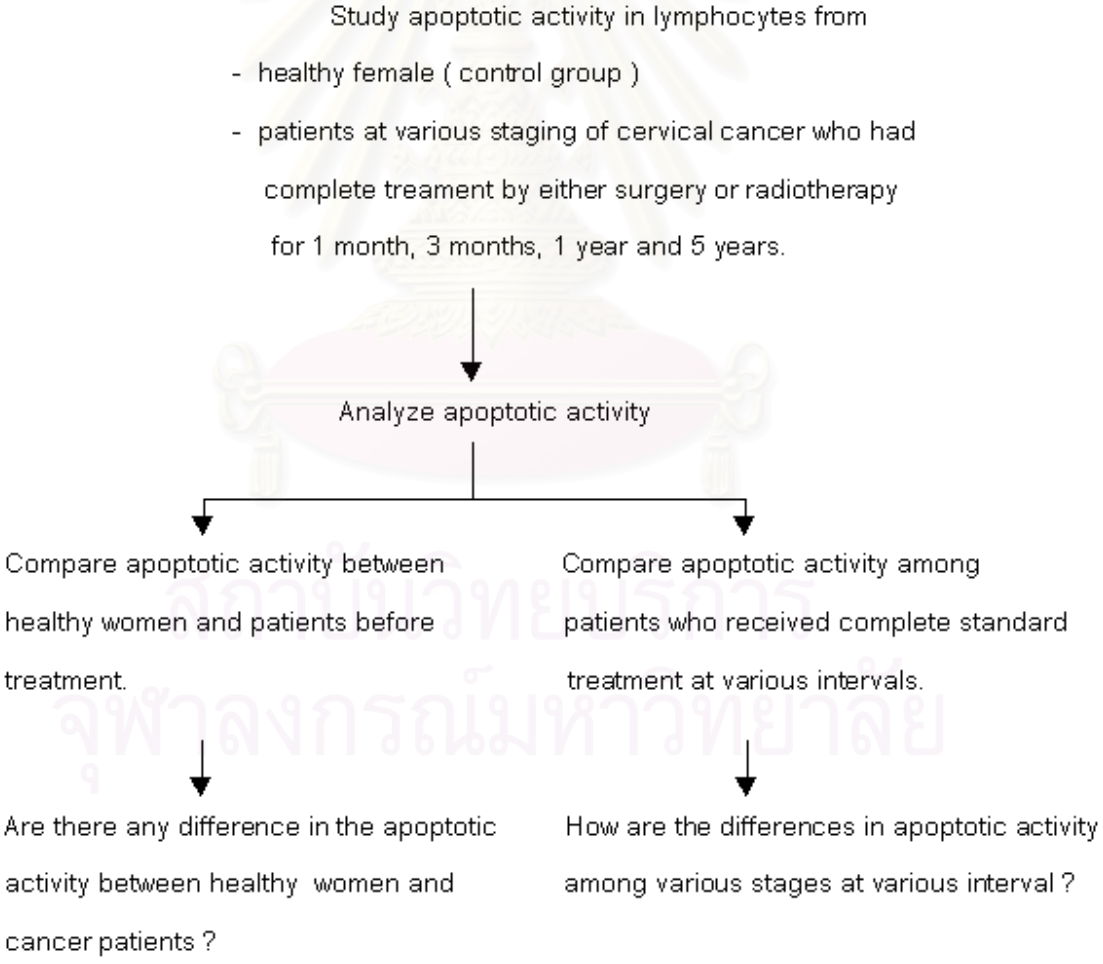
Apoptosis is characterized by nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing. Apoptotic cell is often hard to observe *in vivo* because the dying cells are rapidly phagocytosed by macrophages. Apoptosis is usually associated with DNA fragmentations (sized 50-300 kb.) which are sensitive for *in vitro* detection by various methods: TdT assay, FADU assay, comet assay, and electrophoresis gel etc.[4-6] White blood cell is a nuclear hematocyte which is easily collected from the body. The mononuclear white blood cell can be used as a model for studying apoptosis. Exposure of mononuclear cell to low dose radiation induction can be detected by TdT assay.[5,7] In this study, we used lymphocytes from cervical cancer patients receiving radiation therapy and healthy women for assessing the apoptotic activity.

2. Objectives

1.1 To compare the radiation-induced apoptotic activity of lymphocytes from cervical cancer patients with age matched healthy females.

1.2 To compare the apoptotic activity of lymphocytes from patients with various staging of cervical cancer at 1 month, 3 months, 1 year and 5 years after completion of the standard treatment regimen (surgery, radiation or combined)

3. Conceptual Framework



4. Assumption

Since it is not possible to do prospectively follow up of apoptotic changes in a healthy women and through tumourigenesis till the appearance of cancer lesion. This study is carried out by a cross section design using patients with various stage of cervical cancer and at various time interval after the completion of treatment. The apoptotic activity of these patients are analyzed and compared with apoptotic activity from normal blood donor.

5. Key words

Apoptosis

Cervical carcinoma

Radiation therapy

6. Operational Definition

$$\text{Apoptotic Index} = \frac{\text{The number of apoptotic cells}}{\text{The total number of cells}} \times 100$$

$$\text{Apoptotic activity} = \textbf{Apoptotic Index of irradiation group} - \textbf{Apoptotic Index of control group (not radiated by 0.5 Gy Co)}$$

7. Expected Benefit & Application

1. Establish a base line data on apoptotic activity for healthy Thai women.
2. Establish data base on changes in apoptotic activity that may explain another mechanism of cervical cancer tumourigenesis.
3. Develop a model laboratory which can be applied to detect apoptotic activity induced by toxic agents, chemical agents, or drugs.

8. Research Methodology

- Sample collection

twenty ml of whole blood were collected in heparinized vaccutainer from individual cervical cancer patient and healthy woman with informed consent.

- Process of study

Step 1. The blood was centrifuged in Histopaque to separate lymphocytes out. The lymphocytes were cultured in RPMI 1640 enriched with 5.2 % Fetal Bovine Serum, L-Glutamine, and Gentamycin. Incubated in CO_2 incubator at 37 ^{o}C, 97 % humidity, and 0.3 % CO_2 for 15 minutes.

Step 2. The lymphocytes from step 1 were separated into 2 aliquots

1. Control portion (Not radiated by Cobalt-gamma ray)
2. Radiation treated portion (Irradiated by Cobalt-gamma ray)

The two aliquots were re-incubated in CO_2 incubator. Then, the cells were harvested at 24 hours and 48 hours into 1 % Paraformaldehyde fixative and stored at 4 ^{o}C.

Step 3. The apoptotic cell were fixed on slides and detected with Apoptag Kit by *in situ* terminal deoxynucleotidyl transferase (TdT assay) . Then, the cells were cover stained with Hoechst dye solution, added antifade solution. The slides were covered with a standard glass coverslip and kept in the dark box at -20 ^{o}C.

Step 4. The slides were viewed under a fluorescence microscope for monitoring apoptotic cells.

Step 5. The apoptotic index and apoptotic activity were analyzed.

CHAPTER II
THEORY AND LITERATURE REVIEW

Apoptosis, also known as programmed cell death (PCD), is a form of cell death in which the dying cell plays an active part in its own demise. The term apoptosis was coined in 1972 by Kerr, Wyllie, and Currie. In their report, they are contended that the development of this distinctive type of necrosis, which has previously been called shrinkage necrosis on morphological grounds. Apoptosis can be triggered by noxious agent, and often appears spontaneously or in response to physiological stimuli. They suggest that it should be called " apoptosis " which is used in Greek to describe the " dropping off " or " falling off " of petals from flowers or leaves from trees. (The word is usually pronounced " APP-oh-TOE-sis " with the second " p " remaining silent) [8-11]

Apoptosis is distinct from accidental cell death (necrosis). Numerous morphological and biochemical differences that distinguish apoptosis from necrosis cell death are summarized in Table 2.[12]

Table 2 Types of cell death [12]

Apoptosis	Necrosis
● **Morphologic criteria**	
Deletion of single cell	Death of cell group
Membrane blebbing, but no loss of integrity	Loss of membrane integrity
Cell shrink, ultimately forming apoptosis bodies	Cell swell and lyse
	Significant inflammatory response
No inflammatory response	Phagocytosis by macrophages
Phagocytosis by adjacent normal cell ,and some macrophages	

Lysosomes intact	Lysosomal leakage
Compaction of chromatin into uniformly dense masses	Clumpy, ill-defined aggregration of chromatin

- **Biochemical Criteria**

Induced by physiological stimuli disturbances	Evoked by nonphysiological disturbances
Tightly regulated process with synthetic and Activation steps	Loss of regulation of ion homeostasis
Requires energy	No requirement for energy
Requires macromolecular synthesis	No requirement for protein or nucleic acid synthesis
De novo gene transcription	No new gene transcription
Nonrandom oligonucleosomal length fragmentation of DNA	Random digestion of DNA

The development of multicellular organisms depend on the elimination of selected cells through apoptosis which controlled the number of cells is balance among growth, proliferation and death. Previous study showed that *Caenorhabditis elegans* eliminates precisely 131 cells of its initial 1,090 cells as its hermaphroditic form mature into an adult. Spontaneous apoptosis that occurs in animals and mammals such as a tadpole deletes its tail cell to be a frog. Human embryos use apoptosis to remove webbing between digits. In adult mammals, apoptosis occurs continuously in slow proliferating cell populations, such as the epithelium of liver, prostate, and adrenal cortex, and in rapid proliferating population, such as the epithelium lining of intestinal crypts and the differentiating spermatogonia. When the cells of the uterine wall die and are sloughed off during menstruation, they perish by apoptosis. Skin cells begin life in the deepest layers and then migrate to the surface undergoing apoptosis along the way.[10,13]

Genetic studies in the nematode *C. elegans* showed that there are three important genes (CED-3, CED-4, CED-9) involved in controlling apoptosis during the development of worm. CED-3 and CED-4 promote apoptosis whereas CED-9 inhibits apoptosis. CED-4 binds to CED-3 and promotes the activation of CED-3 , whereas CED-9 binds to CED-4 and prevents it from activating CED-3. Normally, CED-9 is complexed with CED-4 and CED-3, keeping CED-3 inactive.[14-16]

Human homologues of CED-3 gene encode members of the caspase family of proteases. Caspase, a family of cysteine proteases with aspartate substate specificity, is produced in cell as catalytically inactive zymogens which becomes activated by another caspases or by an autocatalytic mechanism, and the active caspase can in turn activate other caspases resulting in a cascade process.[17-18] CED-9 is homologue of the mammalian Bcl-2 family of antiapoptosis. Bcl-2 family includes two subgroups of proteins that either inhibit (Bcl-2, Bcl-x_L, Bcl-w) or promote (Bax, Bak, Bid, Bad) apoptosis.[19 ,20] The human homologue of CED-4 is Apaf-1 (*A*poptosis *a*ctivating *f*actor 1) which is required for the activation of caspase-3. Two Apafs have been identified : Apaf-2 is cytochrom c, and Apaf-3 is caspase-9.[21]

Apoptotic death can be triggered by a variety of stimuli, and not all cells necessarily will die in response to the same stimulus. Any agent or set of condition that stresses the metabolism or normal response mechanisms of a cell is likely to trigger the process of apoptosis. However, the level of stress is crucial. At high stress levels, cell died by necrosis because they have no time to respond to the stimulus and die instantly. Examples include high levels of toxins, sharp change in pH and high agitation rates. At intermediate levels of cell stress, the cell is injured but not killed. The cell has time to activate its own death programme. Thus the cell dies in a controlled way, by apoptosis. At low levels of environmental stress, cell can switch on the production of heat shock proteins which enable them to survive until the stress is removed. However, once a certain stress threshold is passed and survival is deemed impossible. Cell died by apoptosis. [8]

Trigger of apoptosis include deprivation of growth factors, presence of receptor-ligand complexes on the cell surface, toxins, hyperthermia, viruses, free radicals,

irradiation, and chemotherapeutic drugs. These signals can be divided as being external and internal signal.[21]

The external signal is the presence of a family of receptors belonging to the tumor necrosis factor (TNF) receptor superfamily such as the Fas antigen, also known as APO-1/CD95.[22] Fas is a cell surface protein that has been shown to initiate a signal for apoptosis when cross linked with Ligand (FasL) or specific antibody.[23] FasL is a homotrimeric molecule and each FasL trimer binds three Fas molecules.[24] Because death domains have a propensity to associate with one another, Fas ligation leads to clustering of the receptors' death domain[25] (Figure 5) ; which is supported by nuclear magnetic resonance structure analysis studied. An adapter protein called FADD (Fas-associated death domain ; also called Mort1) then binds through its own death domain (DD) to the clustered receptor death domain. FADD also contains a death effector domain (DED) that binds to an analogous domain repeated in tandem within the zymogen form of procaspase-8 (also called FLICE, or MACH).[26] The death effector domain is a specific example CARD (caspase recruitment domain), which is found in several caspase with large prodomain, including caspase-2,-8,-9,-10 upon recruitment FADD, caspase-8 oligomerization drives its activation through self-cleavage. Caspase-8 activates downstream effector caspases such as procaspase-9 committing the cell to apoptosis.[17]

In TNF-induced apoptosis, TNF is produced mainly by activated macrophages and T cells in response to infection. In some cell types, TNF also induced apoptosis through TNFR1 by TNF trimerizes TNFR1 upon binding, including association of the receptors'death domain. An adaptor termed TRADD (TNFR-associated death domain)binds through its domain to the clustered receptor death domains.[27] TNFR1-TRADD complex can binds to FADD to activate procaspase-8, thereby initiating apoptosis.[17] Besides FADD, TNFR1 can engage an adapter called RAIDD (RIP-associated ICH-1/CED-3 homologous protein with a death domain). RAIDD binds through a death domain to the death domain of RIP (receptor-interacting protein) and through a CARD motif to a similar sequence in the death effector caspase-2, thereby inducing apoptosis.[28]

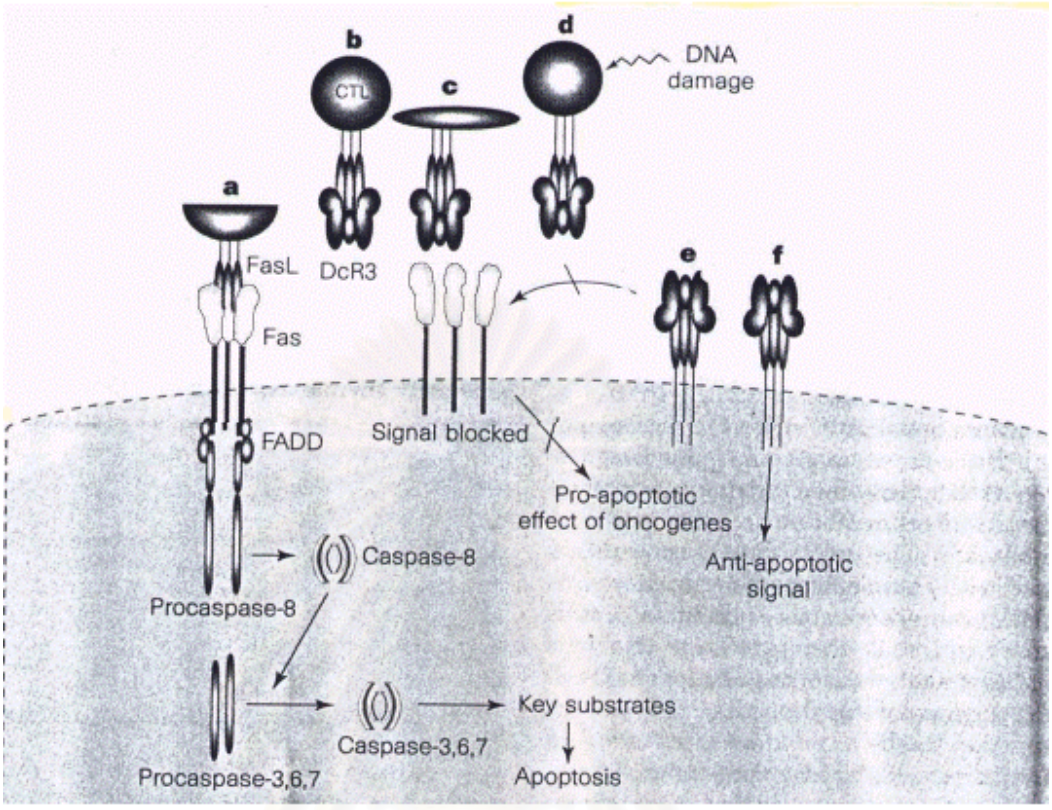


Figure 5 Apoptotic signaling ; (a) Normal, trimerization of FasL – bound Fas recruits the cytoplasmic adaptor molecule FADD which, in turn, recruits procaspase-8, the precusor of an initiator caspase (cystein protease with aspartic- acid specific) involved in apoptosis. Two caspase-8 molecules process one another, and assemble to form the mature, active caspase. This cleaves and activates other caspase, which then orchestrate apoptotic cell death. (b – f) Anti-tumorigenesis measure that may be blocked by the binding of DcR3 to FasL. (b) Cytotoxic lymphocytes (CTLs) express FasL. (c) Surrounding cell express FasL in response to tumor infiltration. d) DNA damage by therapeutic agents induces expression of FasL on tumour cells, which may kill neighboring cells. (e) Fas/FasL signaling normally contributes to the pro-apoptotic effects of oncogenes (such as c-Myc). (F) Ligation of FasL delivers an anti-apoptotic signal to the cell.[22]

Decoy receptor 3 (DR3) shows close sequence similarity to TNFR1. It can binds to Apo3L, which is related most closely to TNF, and then trigger apoptosis through TRADD and FADD to activate caspase cascade. Apo3L-DR3 and TNF-TNFR1 interactions probably have distinct biological roles. [17]

The internal signal of apoptosis (due to oxidative stress, irradiation or presence of virus, toxin, chemotherapy drug) surprisingly occurs through the release of cytochome c from the mitochondria. The permeability of mitochondrial membranes is a critical event that results in the release of cytochome c (a caspase activator), adenosine triphosphate (ATP), Smac/Diablo (a caspase coactivator), and an apoptosis-inducing factor, which activate the nucleases that damaged DNA into small fragment.[29-31]

An important question is how mitochondria are able to respond to intracellular signals and release cytochome c. A strong candidate for the signal decoder is a mitochondrial ion channel called mitochondrial permeability transition pore (MPT). MPT appears to be a multisubunit structure that contains the mitochondrial ATP transporter and a voltage-dependent anion channel as well as other protein. It is located at the point where the inner and outer mitochondrial membranes are closely apposed. Presence of oxidants and pathological level of Ca^{2+} cause MTP to open into an irreversible high-conductance state that results in collapse of the mitochondrial membrane potential and release of cytochome c between the two membranes (Figure 6) . [21]

A potential proapoptotic transcription factor, TR3 (also called NUR77 or NGFIB) ,is a known outer mitochondrial membrane protein, normally present in the nucleus, but it can move to mitochondrial surface. TR3 may also induce mitochondrial permeability to induce cytochome c release and apoptosis.[29,30,32] The Bcl-2/Bax/Bid family proteins has been reported that it can translocated to mitochondria (Figure 7). These proteins permeabilize the outer mitochondrial membrane upon interaction with the permeability transition pore complex (for example, Bax), or altenatively, independently of such an

interaction (for example, Bid). Intriguingly, it was recently found that p53 moves from the nucleus to the mitochondria where it interact with hsp70 (a heat shock protein), a mitochondria-specific protein. The impact of the interaction between p53 and hsp70 on mitochondrial membrane integrity has not yet been elucidated.[29]

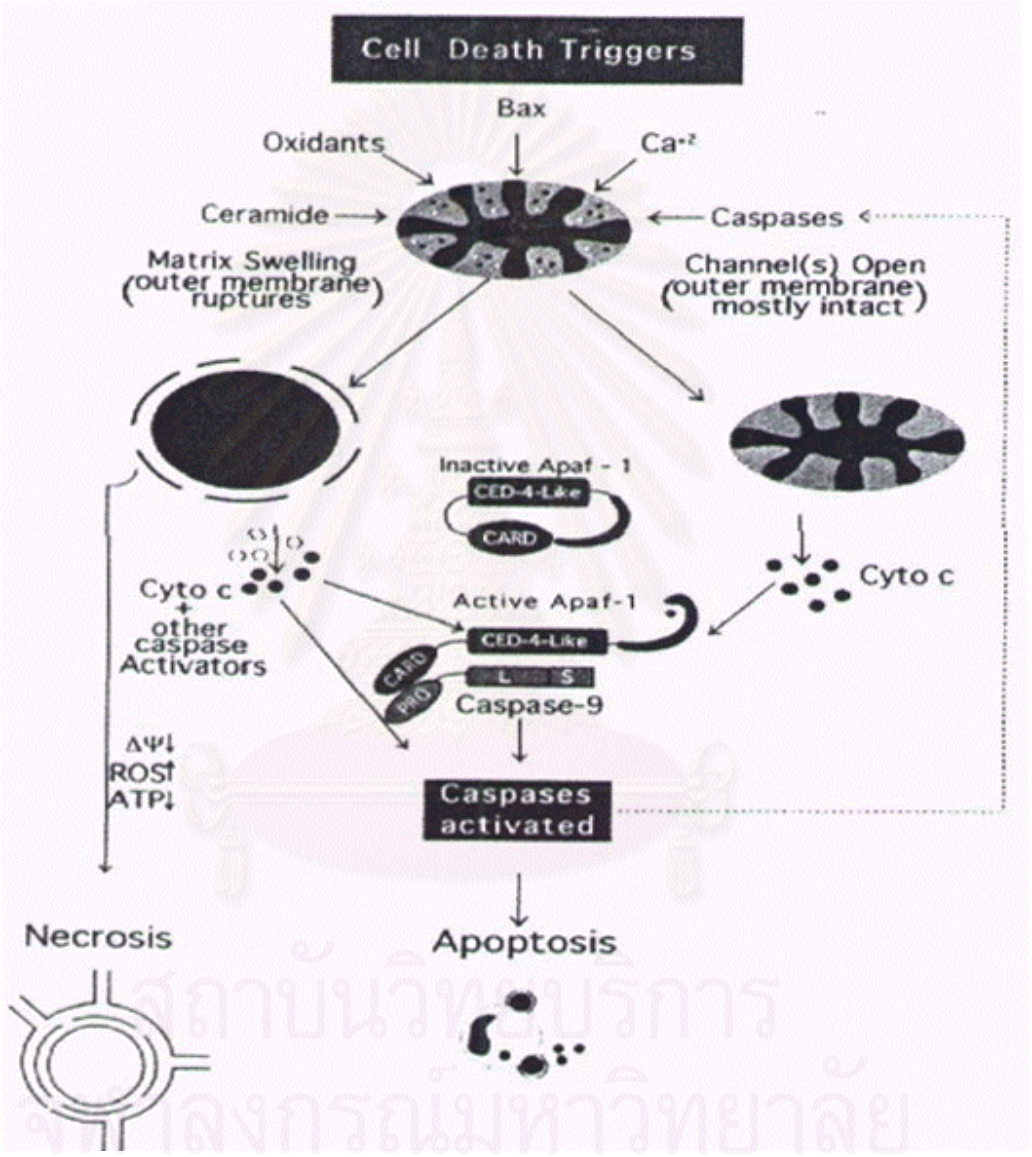


Figure 6 Model for caspase activation by mitochondria. Release of cytochome c into the cytosol results in Apaf-1 activation and subsequent activation of pro-caspase-9 followed by downstream effecting caspases which induce apoptosis.[30]

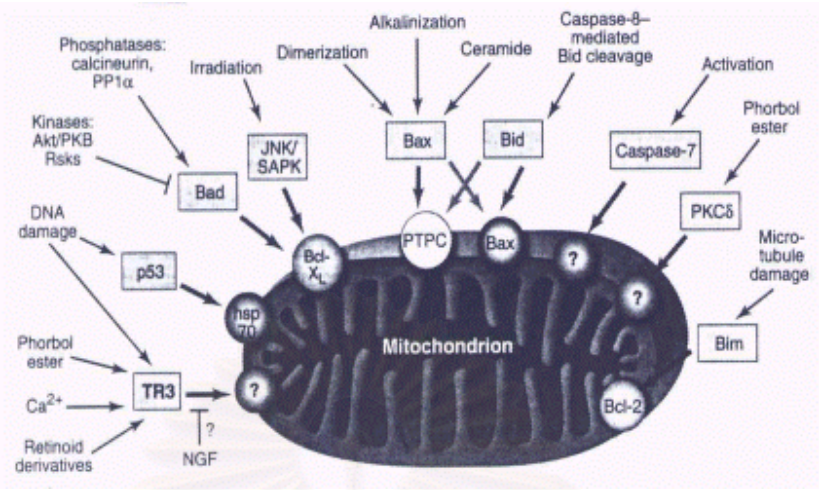


Figure 7 Protein that move to and affect mitochondrial membranes.[29]

In the cytosol, cytochrome c associates with Apaf-1 in the presence of dATP or ATP and induce its oligomerization. The oligomeric Apaf-1 complex recognizes the inactive procaspase-9 and-3, forming the "apoptosome" , which induces autocatalytic processing of procaspase-9. The mature caspase-9 in turn activates its primary downstream target procaspase-3.[16,29]

Whatever the pathway of signaling, the majority of responses occurs through activation of caspase-3. Procaspase-3 can be activated by caspase-8 (external signal pathway) or by caspase-9 (internal signal pathway). In turn caspase-3 can activate other caspase (caspase-6, -9), through proteolytic cleavage at specific internal Asp residues (Asp-x-x-Asp, where x is any amino acid). [29,33,34] Once activated, the effector caspases are responsible for proteolytic cleavage of a range of cellular targets, ultimately leading to cell death. Summary of trigger apoptosis was showed in Figure 8.

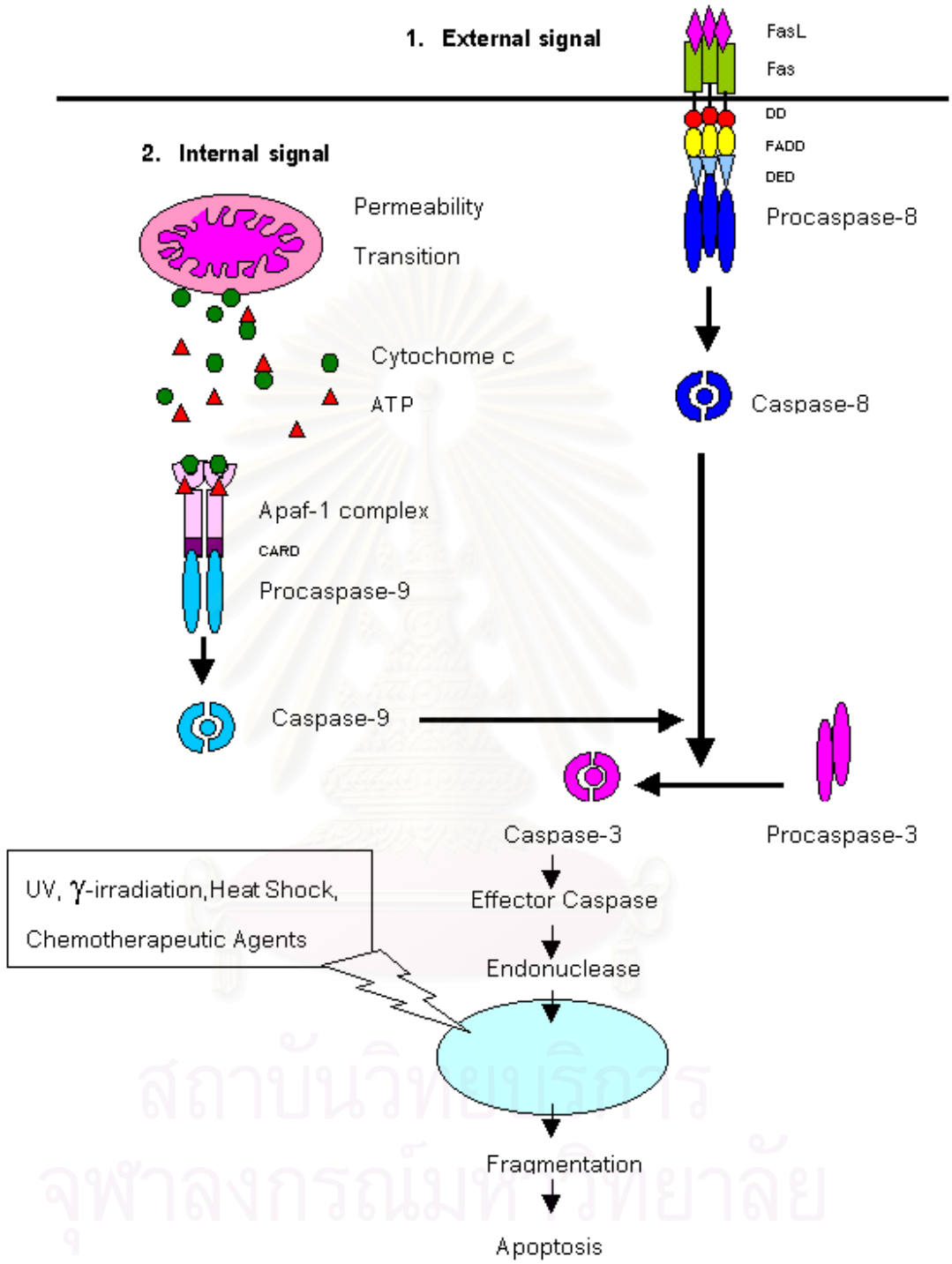


Figure 8 Trigger apoptotic signal

Apoptosis is usually associated with the activation of endonucleases that degrade the chromosomal DNA first into large (50 to 300 kilobases) and subsequently into very small oligonucleosomal fragments.[8,10] Proteolysis of fodrin and actin can account for change on the cell outer surface (blebbing) and that of nuclear lamins for the nuclear fragmentation.[21] During apoptosis, caspases contribute to apoptosis through direct disassembly of cell structure, as illustrated by the destruction of nuclear lamina, which is mainly in the nuclear membrane and is involved in chromatin organization. Lamina is formed by head-to-tail polymers of intermediate filament proteins called lamins. During apoptotic process, lamins are cleaved at a site by caspase, causing lamina to collapse and contributing to chromatin condensation. Caspase also recognizes cell structure indirectly by cleaving several proteins involved in cytoskeleton regulation including gelsolin, focal adhesion kinase (FAK), and p-21-activated kinase 2 (PAK2).[18]

At the final step of apoptotic process, the asymmetry of plasma membrane phospholipids is lost causing the exposure of phosphatidylserine (PS), normally localized at the inner leaflet of the plasma membrane. Appearance of phosphatidylserine on the outer leaflet of the plasma membrane is specifically recognized to uptake and degradation by macrophages and semi-professional phagocytes, which is important function in the downregulation of inflammatory response after uptake of apoptotic cells by macrophages (Figure 9).[35,36]

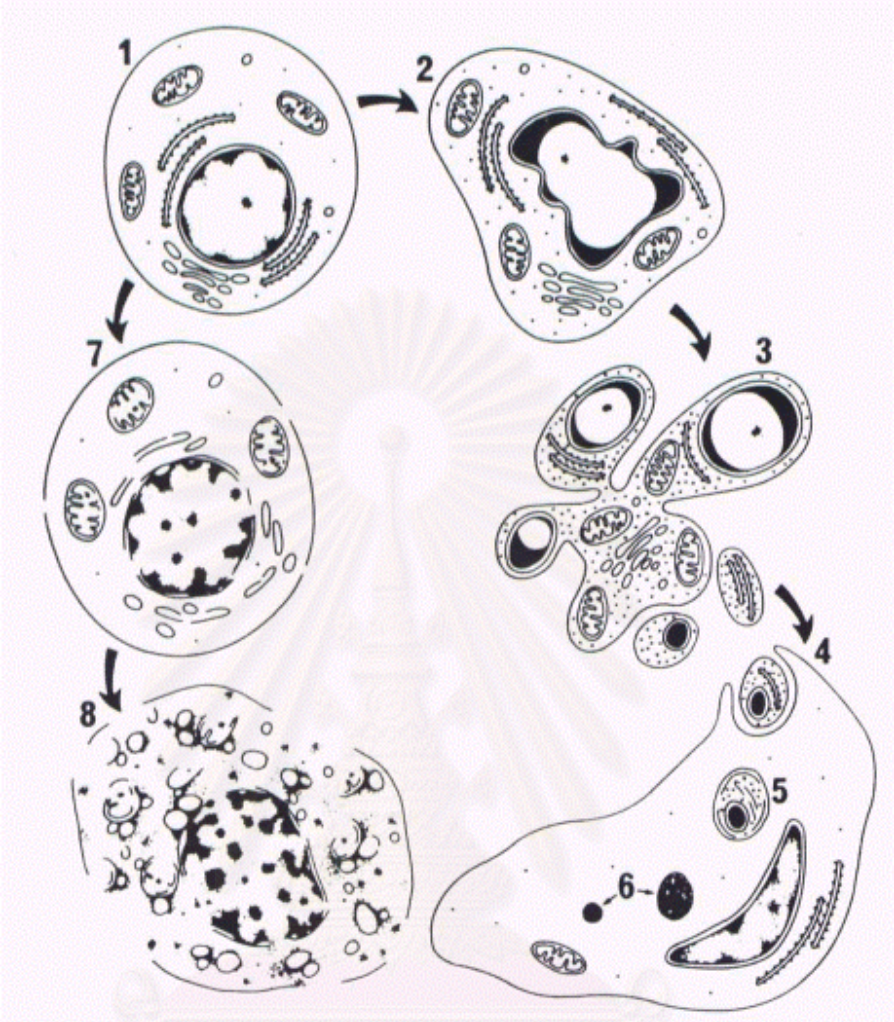


Figure 9 Diagram illustrating sequence of ultrastructural changes in apoptotsis (2-6) and necrosis (7 and 8). (1) Normal cell. Early apoptosis (2) is characterized by compaction and margination of nuclear and chromatin, condensation of cytoplasm, and convolution of nuclear and cell outlines. (3) At the last stage, the nuclei fragments and protuberances that form on the cell surface separate into apoptotic bodies, which (4) are phagocytosed by nearby cells and (5 and 6) degraded within lysosome. (7) The development of necrosis is associated with irregular clumping of chromatin, marked swelling of organelles and focal disruption of membranes. (8) Membranes subsequently disintegrate, but the cell usually retains its overall shape until removal by mononuclear phagocytes.[10]

Inhibitor of apoptotic family proteins

Inhibitor of apoptotic (IAP) family proteins constitute a group of apoptotic suppressors which are conserved throughout animal evolution, with homologues in flies (*Drosophila*), worms (*C. elegans*), mice and humans. These proteins function predominantly as direct inhibitors of certain caspases (Figure 10).[37,38]

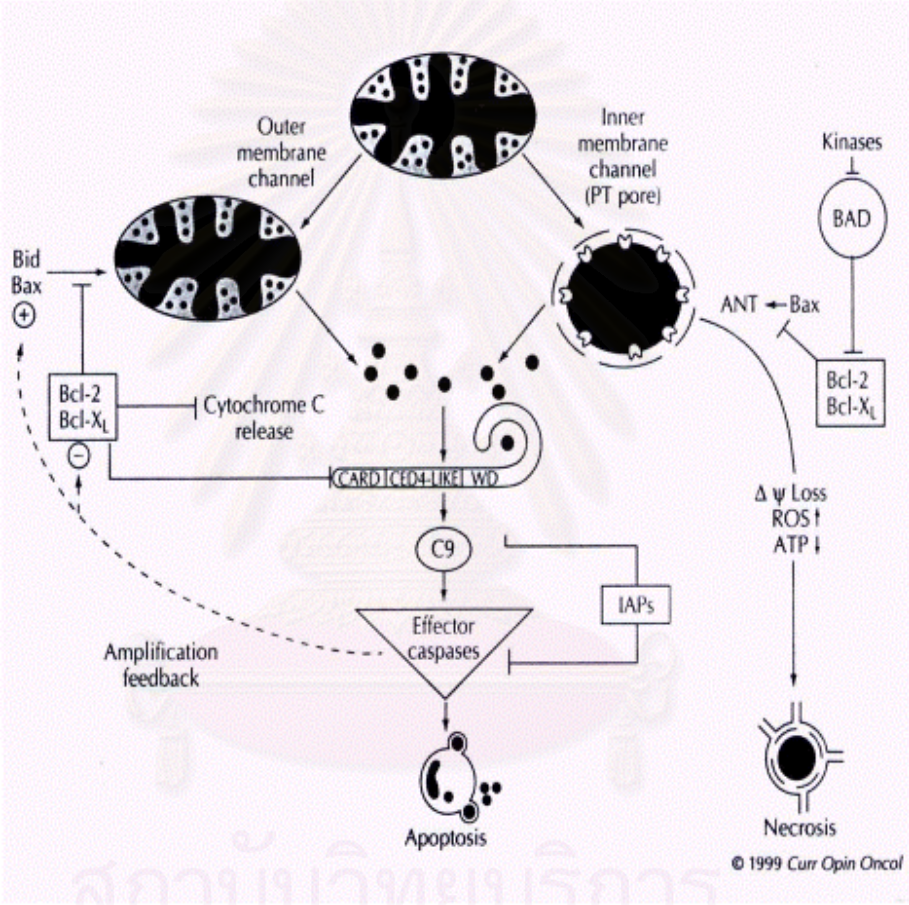


Figure 10 Model for IAPs family inhibits apoptotic process . The release of cytochome c is blocked by Bcl- 2 . However, Bcl- 2 or its homologues such as Bcl-x also bind Apaf-1 and thwart its activation of caspases.[37]

Only caspase-3, -7,-9 have been reported to be bound and inhibited by IAPs. Caspase-1,-6,-8, and -10 are not. Thus, the caspases that are inhibited by IAPs operate mostly in the distal portions of apoptosis proteolytic cascades (eg, caspase-3, and -7), functioning as ultimate effectors of apoptosis by cleaving various proteins responsible for cell death. The expression of most IAP family protein in either normal human tissues or tumors is not clear. However, one of the major IAP family is represented by Bcl-2 and its homologues. The human Bcl-2 family proteins including Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1/Bfl-1 suppress apoptosis.[21,37] The pathological levels of one or more of these IAP proteins have been observed in several types of cancer, but the mechanisms for their increased expression are poorly understood.[37,39,40]

Previous study showed that the ratio of Bcl-2 to Bax determines the occurrence of apoptosis after an apoptotic stimulus. That is a high of Bax-Bax homodimer than Bax-Bcl-2 heterodimers shifts the balance to promotion of apoptotic cell death, and a higher ratio of Bcl-2-Bcl-2 homodimers than Bax-Bcl-2 heterodimers shifts the balance to promotion of cell survival. In addition, Bcl-2 and Bax proteins that are known to regulate irradiation-induced apoptosis are expressed in normal and neoplastic epithelium of the uterine cervix.[41-44] Radiation induced apoptosis was associated with Bax protein expression in cervical carcinoma. Bax expression was a significantly increased by irradiation, and there was a significant correlation between Bax expression and apoptosis.[45,46]

One important activity of Bax and Bcl-2 is the involvement in the release cytochrome c from mitochondria. Several apoptotic stimuli induce translocation of the pro-apoptosis proteins such as Bax from cytosol to mitochondria, where they induce release of the cytochrome c, but it is inhibited by the presence of Bcl-2.[20,30] Bax protein inducing cytochrome c release from mitochrondria hypothesizes a Bax-falcilitated opening of the ANT (Adenine nucleotide translocator) , a channel protein normally involved in ATP and ADP exchange across the inner membrane of mitochrondria that cause osmotic dysequilibrium of mitochrondria. Bcl-2 and Bcl-X_L, which clearly have ion-channel activity

in vitro, prevent mitochondrial permeability transition pore opening and cytochrome c release.[37] The only known mechanism for activating Apaf-1 is cytochrome c, which binds to Apaf-1. Bcl-X_L reportedly can bind Apaf-1, suggesting Bcl-X_L interfered with the activation of caspase by cytochrome c and Apaf-1.[47]

The Bcl-2 family gene are regulated by p53. The p53 gene regulates the expression of genes such as MDM2, p21 and Bax gene.[44] In addition, p53 is a capable drive for the process of apoptosis. The function of p53 is to arrest cell in the G1 phase of the cell cycle. This allows time for cell to repair the damage before DNA synthesis occured. If the damage cannot be repaired, then apoptosis is triggered. In addition, if p53 fail to perform its duties, perhaps due to mutation, then cells with genetic damage can re-enter the cell cycle possibly giving rise to cancer.[8,10] *c-myc*, like *p53*, encodes Myc protein, that is expressed in proliferating cell but is absent in quiescent cells. Myc drives both the process of cell proliferation and cell death. The presence of Myc and survival signals induce cell proliferation. In the absent of survival signal, apoptosis is triggered. Myc promotes apoptosis by a nontranscriptional mechanism.[34]

Survivin, inhibitor of apoptosis protein, is expressed in a large proportion of human cancer, providing evidence that altered expression of these proteins can occur during tumorigenesis.[37] In addition, survivin can inhibits the terminal effectors caspase - 3 and -7 too.[48]

Apoptosis and Disease

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. Recent evidences suggest that the failure of cells to undergo apoptosis might be involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune disease, viral infections. Moreover, a wide number of disease characterized by cell loss, such as neurodegenerative disorders, AIDS (acquired immunodeficiency syndrome) , and osteoporosis, may result from accelerated rates of physiological cell death.[9,49]

Table 3 Diseases associated with imbalanced apoptosis [49]

● Disease Associated with the Inhibition of Apoptosis	● Disease Associated with Increased Apoptosis
1. Cancer	1. AIDS
Follicular lymphomas	2. Neurodegenerative disorders
Carcinomas with p53 mutations	Alzheimer's disease
Hormone-dependent tumours	Parkinson's disease
Breast cancer	Amyotrophic lateral sclerosis
Prostate cancer	Retinitis pigmentosa
Ovarian cancer	Cerebellar degeneration
2. Autoimmune disorders	3. Myelodysplastic syndromes
Systemic lupus erythematosus	Aplastic anemia
Immune-mediated glomerulonephritis	4. Ischemia injury
3. Viral infections	Myocardial infarction
Herpesviruses	Stroke
Poxviruses	Reperfusion injury
Adenoviruses	5. Toxin-induced liver disease
	Alcohol

Disease is characterized by the accumulation of cells include cancer, autoimmune disease, and certain viral illnesses. Cell accumulation can result from either increased proliferation or the failure of cells to undergo apoptosis in response to appropriate stimuli. Although much attention has focused on the potential role of cell proliferation in these diseases, increasing evidence suggests that alterations in the control of cell survival are important in the pathogenesis of these so-called proliferative disorders.[9,49,50]

● **Apoptosis in cancer**

Cancer develops after cell accumulates mutations in several genes that control cell growth and survival. When the mutation seems irreparable, the affected cell usually kills itself rather than risk becoming deranged and potentially dangerous. If the cell does not die, it or its progeny may live long enough to accumulate mutations that make it possible to divide and metastasize to break away from the original tumor and establish masses at a distant sites. Some cancer cells divide more slowly than normal cells, but the cancer may still expand because of prolonged cell lifespan.[50] Many DNA damage cell can respond to sort of injury in several ways : it can delay cell division until the damage is repaired, it can undergo apoptosis, or it can progress without interruption through the cell growth cycle. Apoptosis is an efficient process for preventing malignant transformation because it removes cell with genetic lesion. Abnormal apoptosis can promote cancer development, both by allowing accumulation of dividing cell and by obstructing removal of genetic variants with enhanced malignant potential. [9,49,50] Many human cancers have mutations or deletions in the *p53* gene.[51] Malfunctions of *p53* may promote cancer development by permitting cells with secondary mutations to duplicate their DNA before the repaired process is completed. However, *p53* is not just an inhibitor of cell division ; in other situations it acts as a direct " apoptogene " - a gene that causes apoptosis. Overproduction of normal *p53* protein in a myeloid leukemia cell line induces rapid cell death by apoptosis.[52] Study on apoptosis has began to clarify why many tumors are resistant to treatment. Researchers studied on genes and proteins that correlate with cancer to search for ways to prevent overactive oncogenes and to induce apoptotic genes.

- **Apoptosis in autoimmune disease**

Under normal conditions of the immune system, apoptosis has are important roles. For example, apoptosis reduces the autoreaction T-cells in the thymus that is responsible for self-tolerance and selects B-cells in lymphoid germinal centers during humoral immune response. So a defect in the deletion of autoreactive lymphocytes by apoptosis can predispose to autoimmunity.[9,50] Some evidence in animals and humans indicates that extended survival of autoreactive cells is implicated in at least two chronic autoimmune syndrome : systemic lupus erythematosus and rheumatoid arthritis.[9] Patients with systemic lupus erythematosus have elevated levels of soluble Fas, which may competitively inhibit FasL-Fas interaction. The resulting decrease in Fas-mediated apoptosis may contribute to the accumulation of autoimmune cells in this disease.[49]

- **Apoptosis in viral infection**

When viruses enter the cell, they attempt to shut down that cell's ability to make any proteins except those needed to produce more viruses. Virus inhibits host protein synthesis that is essential for inducing many kind of cell to commit apoptosis. If the host cell dies, the virus is eliminated. Therefore, certain viruses have evolved ways to inhibit apoptosis in the cells that they infect.[9]

The prevention of apoptosis is also important for the establishment of viral latency. Epstein-Barr virus establishes a latent infection in B cells. The viral gene LMP-1, which is produced during latency, specifically up-regulates the expression of Bcl-2, potentially providing a survival advantage to latently infected cells. Chronic Sindbis virus infection has also been reported to be dependent on the host cell's expression of Bcl-2.[49] Papillomavirus, a major case of cervical cancer, inactivates or degrade the apoptotic inducer p53. Cowpox virus, a relative of which is used as the smallpox vaccine, elaborates a protein that prevents ICE-like proteases from carrying out the apoptosis program, suggesting that some

human viruses may do the same. This is interesting in antivirus therapy which is exploring ways to block the activity of the antiapoptosis molecules manufactured by viruses. [9]

Excessive cell death can result from acquired or genetic condition that enhance the accumulation of signal that induce apoptosis or that decrease the threshold at which such events induce apoptosis. Although increased apoptotic cell death has been observed in many of the diseases discussed below.

- **Apoptosis in AIDS**

In individuals with human immunodeficiency virus 1 (HIV-1) infection, depletion of CD4 T cells leads to lymphopenia and immunodeficiency. AIDS may be regarded as a pathological imbalance between the rate of CD4 cell death and cell replacement.[50] It has been shown that CD4 acts as a receptor for viral attachment facilitating HIV infection of CD4 T cells. Surprisingly, most T cell that dies during HIV infections do not appear to be infected with HIV. Recent evidence suggests that stimulation of the CD4 receptor by its binding to HIV-1 gp120 glycoprotein, results in the enhanced susceptibility of uninfected T cells to undergo apoptosis.[49,50] If apoptosis really is a mechanism of lymphocyte depletion in AIDS, blocking an essential metabolic step in apoptosis might delay the onset of immunodeficiency.

- **Apoptosis in neurodegenerative disorders**

A wide variety of neurological disease are characterized by the gradual loss of specific sets of neurons, such as Alzleimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis (ALS), Retinitis pigmentosa, Spinal muscular atrophy, and various forms of cerebellar degeneration. In these disease, cell death results in specific disorders of movement and central nervous system function. The cell loss in these disease does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell

death. Oxidative stress, calcium toxicity, mitochondrial defects, excitatory toxicity, and deficiency of survival factors have all been postulated to contribute to the pathogenesis of these disorder.[49]

Alzheimer's disease is associated with the progressive accumulation of β-amyloid (Aβ) peptide in plagues. Mutation in the β-amyloid precursor protein are associated with some forms of familial Alzheimer's disease. Recently, several groups have shown that β-amyloid peptide induces neurons to undergo apoptosis,[49,50] and researchers have found that the brains of Alzheimer's patients contain dying neurons that display certain characteristic signs of apoptosis, such a DNA break.[53]

Apoptosis in cell culture system

Animal cell cultures are useful for the development in biotechnology and phamaceutical industries for the production of diagnostic and therapeutic proteins. Researchers develop strategies to optimize the culture conditions for cell growth. It is important to use a medium which can reduce the proliferation of cell cultures and induce cell death by apoptosis.[12] Serum and growth factors in medium are essential for apoptotic response.[8] In this study, we used RPMI 1640 medium which is completed by adding 5.2 % fetal bovine serum, L- Glutamine ,and Gentamycin. In addition, apoptosis in cell culture system is induced by radiation, chemotherapeutic agents, mild hyperthermia, hormone withdrawal or addition, antibody, and cytotoxic lymphocytes.[10]

● **Induction of Apoptosis by Radiation**

At present, researcher suggests that DNA is the target of cytotoxic effect of radiation. Radiation can induces apoptosis via several mechanisms: causing DNA damage, ceramide production, generation of the reactive oxygen species. The molecular basic of radiation damage repairing system in mammalian is only partially known. The physical interaction of ionizing radiation with the molecular infrastructure of the cell results in some types of chemical reactions. When any form of radiation (X-rays, γ-rays) is absorbed possibly by biological material, there is a possibility that it will interact directly with the critical target in the cell. The atom of the target itself may be ionized or excited, and initiated a chain of events that leads to a biological change. This is called direct action of radiation. Alternatively, the radiation may interact with other atoms or molecules in the cell such as water molecule to produce free radicals which are able to diffuse far enough to reach and damage the critical target. This is called indirect action of radiation. The indirect action is dominant for ionizing radiation such as X-rays. It is estimated that 75 % of the X-ray damage to DNA in mammalian cells is due to the $OH^{\cdot}$ radical (Figure 11). The step between the breakage of chemical bonds and the expression of the biological effect may be days, months, or years.[54,55] Exposure to ionizing radiation in small to moderate doses induces apoptosis in normal tissues without tissue necrosis.

Moreover, radiation damages to the membrane. It is wildly accepted that, after irradiation with low dose which cause reproductive death, the consequences of membrane damage accompany other radiation-induced change observed at cellular level. γ-irradiation activates acidic sphingomyelinase to produce ceramide, a catabolic product of membrane sphingolipids that is a death signal. Ceramide can also be generated *de novo* by ceramide synthase, as was shown in LNCaP cells induced to under apoptosis after exposure to 12-o-tetradecanoylphorbol-13-acetate. Although ceramide appears to function as a second messenger for strees signalling. There is still some controversy about the precise role that ceramide plays in apoptosis.[56]

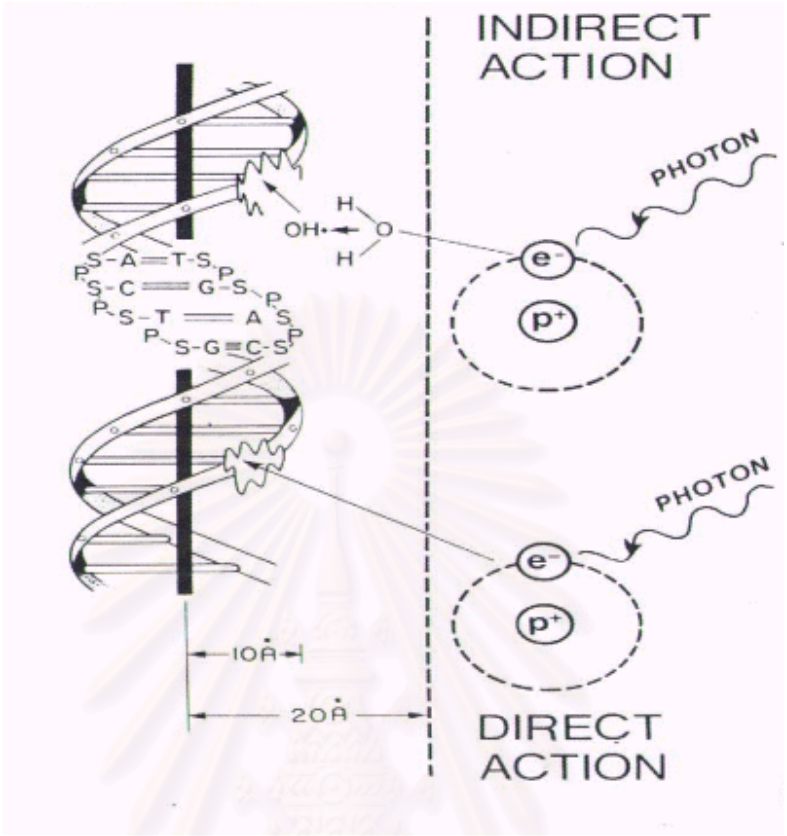


Figure 11 Radiation induces DNA break. Direct action: a secondary electron resulting from absorption of an X-ray photon interacts with the DNA to produce an effect. Indirect action: the second electron interacts with, for example water molecule to produce an $OH^{\bullet}$ radical, which in turn produces the damage to the DNA.[54]

- **Induction of Apoptosis by Cancer Chemotherapeutic Agents**

A variety of anticancer drugs have been shown to induce extensive apoptosis in rapidly proliferating normal cell population, lymphoid tissues, and tumors.[10] The way in which anticancer drugs induce apoptosis is unknown. However, there is evidence that stimulation of some cell lines by trophic cytokines or increase in their level of expression of the bcl-2 proto-oncogene can greatly increase their resistance to the apoptosis-inducing effect of anticancer drugs.[57]

● **Induction of Apoptosis by Mild hyperthermia**

In susceptible tissues, heating to 43 °C for 30 minutes induces extensive apoptosis, whereas heating to temperatures of 46 °C and greater for same periods produces necrosis.[10] Heating at 45 °C induces DNA strand break which cannot be detected immediately after heating, and 37 °C postheating incubation of at least 11.5 hours was required before detectable break occurred.[58]

● **Induction of Apoptosis by Hormone Withdrawal or Addition**

Apoptosis is involved in the atrophy of endocrine-dependent organs, such as prostate and adrenal cortex that follows withdrawal of trophic hormonal stimulation. In contrast, increased levels of glucocorticoid induce apoptosis of thymocytes[59] and similar effect is observed with many lymphocytic leukemias and malignant lymphomas.[60] In addition, Bcl-2 expression has been shown associated with resistant to induction of apoptosis by glucocorticoids in several lymphoid cell lines.[61]

Methods for the detection of apoptosis

Numerous methods of induce apoptosis are used for the induction of apoptosis *in vitro* for scientific studies. There are a number of laboratory procedures available for detecting and quantifying levels of apoptosis in cell cultures. The simplest of these is examination of the morphology of cells that are stained with dyes such as heamatoxylin and eosin.[11,62]

An alternative method that is widely used is extraction and analysis of DNA on agarose electrophoresis gels to detect internucleosomal DNA fragmentation. The DNA samples were electrophoretically separated in 1 % agarose gel containing ethidium

bromide. DNA was visualized with a UV transilluminator, and the gels were photographed. The tell- tale ladder pattern is an indicator of cell death via apoptosis.[63-65] The degree of DNA fragmentation that occurs during the apoptotic process is sensitive for detection apoptotic cells via method such as the *in situ* terminal deoxynucleotidyl transferase (TdT) assay, using digoxigenin labelled nucleotides by TdT reaction at the 3'-OH end of the DNA fragmentation. The incorporated digoxigenin-nucleotides within the cells were detected with fluorescein labeled antidigoxigenin antibodies. The antidigoxigenin antibodies fragment carries a fluorophone (fluorescein) to the reaction site. When excited by light of 494 nm wavelength, the fluorescein generates an intense signal at 523 nm. Finally, the cells were counter stained with 4,6-diamino-2-phenylindole (DAPI). DAPI will stain the nuclei and these fluorescein labeled apoptotic nuclei were detected by fluorescence microscope (Figure 12).[5,63,65,66]

The fluorescence analysis of DNA unwinding (FADU) assay was used to measure DNA single strand breaks, DNA double strand break, alkali-labelled lesion and detect DNA fragmentation which associated with apoptotic nuclei. When double-strand DNA is exposed to moderately alkaline solutions, hydrogen bonds are broken and the 2 strands are unwound. It has been observed that the rate of unwinding of DNA fragments in alkali is increased by prior exposure of cells of DNA unwinding which can be used as a sensitive measure of strand break.[5,6]

Comet assay or the single-cell gel electrophoresis is a simple, rapid and inexpensive method for DNA strand break detection in individual cell. Because apoptosis is characterized by extensive DNA cleavage, this assay has proved useful in detecting apoptotic cells as those in which only a small amount of DNA stays in the original position of the nucleus.[7]

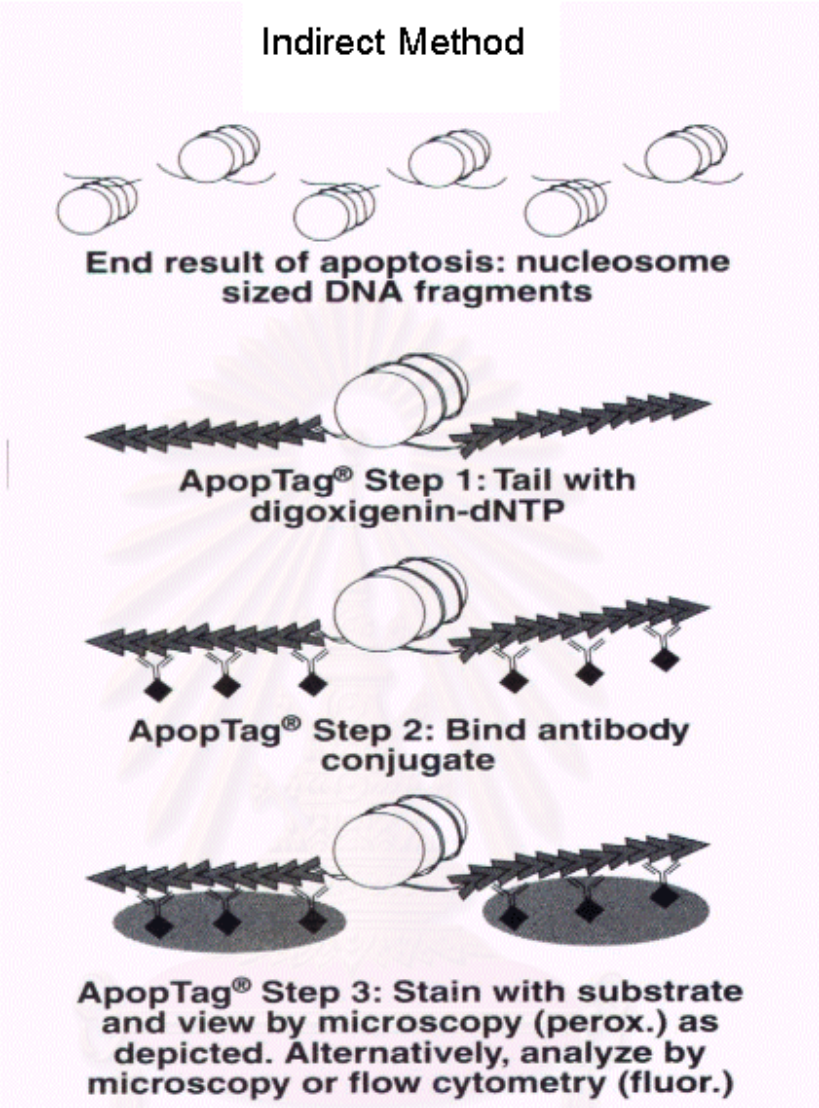


Figure 12 Process of *in situ* terminal deoxynucleotidyl transferase (TdT) assay.[12]

Literature review

The realization that apoptosis occurs in tumors is not new. More than 20 years ago it was suggested that apoptosis may account for much of the spontaneous cell loss known from kinetic studies to occur in many tumors. In addition, apoptosis has been clear for some time that its extent often is enhanced in tumors by well-established treatment modalities, such as irradiation, cytotoxic chemotherapy, heating, and hormone ablation.[9,10,11] However, during the past few years, study involved the radiation-induced apoptosis in tumor tissue is widely carried out.

Ohno et al,[45] assessed the relation between apoptosis and expression of Bax and Bcl-2 protein in fractionated radiation therapy for cervical carcinoma by using *in situ* nick end labeling (ISEL / TdT assay) and immunohistochemical method. Specimens were excised from the same site in cervical tumors before radiation therapy (RT) and at a total dose of 9 Gy. Their results indicated that the mean apoptosis Index (AI) at the dose of 9 Gy was significant increase compared with the mean AI before RT (p =0.0004), and Bax protein expression was significant increase (p =0.013) but not in Bcl-2 protein expression. Thus, Bax protein expression is associated apoptosis induced by fractionated radiation therapy. Bax protein expression appears to play an important role in radiation-induced apoptosis.

Kokawa et al,[43] had studied about apoptosis to support Ohno et al. They studied apoptosis in cervical tissues from cervical cancer patients with invasive squamous cell carcinoma (ISCC) of stage IIIB disease who had received RT. The clinical stage was based on the FIGO criteria. They demonstrated AI before RT, and during RT at the dose of 900, 1980, 3960, 6300 cGy. Apoptotic cells were detected by *in situ* labeling of DNA with dig-ddUTP. The results showed that the AI value at a dose of 900 cGy is significant increase compared with the AI value before RT and at dose of 6300 cGy. Therefore, they

concluded that low dose of irradiation induced apoptosis in ISCC better than high dose of irradiation, and apoptosis was associated with the increased expression of Bax protein but not with increased expression of Bcl-2.

Nunez et al,[67] studied the role of initial DNA damage by examining the radiosensitivity of 2 different types of normal cells from the same patients; epidermal skin cells and lymphocytes, in a paired evaluation. Skin samples were excised from mastectomy specimens from women with breast cancer, and lymphocytes were collected from the same patients and hypersensitive patients. Lymphocytes and epidermal skin cells were estimated initial DNA damage by different methods : (i) by measuring the percentage of DNA different from the well, and (ii) by calculating double strand break (dsb)/Gy/DNA unit. They were estimated molecular radiosensitivity parameter to indicate inter-individual variation in radiosensitivity. The data showed that there is a close relationship between molecular radiosensitivity parameter of initial radiation-induced DNA damage (dsb/Gy/DNA) in both epidermal skin cells and lymphocytes. Thus, the relative radiosensitivity of different cell types taken from the same individual are similar, perhaps reflecting a common genetic determinant of radiosensitivity. They suggest that the predictive value of radiosensitivity assessment in lymphocytes from patients before RT for breast cancer may indicate whether lymphocytes can be used in this way to identify patients who likely to suffer damage to normal tissues.

Wheeler et al,[68] studied apoptosis in pretreatment biopsy specimens from cervical cancer patients with stage IB adenocarcinoma in a retrospective analysis. Their hypothesis is if the spontaneous level of apoptosis plays a similar role in tumor responsiveness to radiation in human, then patients whose pretreatment biopsy specimens exhibit high level of apoptosis should have a better tumor response to treatment with RT than those whose tumors display low level of apoptosis. The specimens were stained with hematoxylin and eosin to detect apoptotic cells. Their results showed that patients whose tumors had a baseline level of apoptosis above the median value (2 %) had a better overall survival than

those with lower levels of apoptosis(p =0.056). So, they concluded that the baseline level of spontaneous apoptosis predicted for survival in patients with stage IB cervical adenocarcinoma. In addition, they suggest that apoptosis may be a marker for the biologic aggressiveness of the tumor, and apoptosis needs to be further evaluated as a potential predictive assay for other human tumors treated with RT.

Tsang et al,[69] studied the relationship between apoptosis and tumor proliferation in human uterine cervix carcinoma II. Tumor proliferation value is a significant cause of failure in the RT of cervix cancer. It is difficult to measure proliferation rate of clonogenic tumor cell during a cause of RT. The specimens from cervix cancer patients who were treated by RT were detected by flowcytometric using the bromodeoxyuridine labelling method, histological , and immunohistochemical analysis. Labelling index (LI) acts as a parameter of tumor proliferation. Their results showed that tumor size was a strong and highly significant factor in predicting survival of cervix carcinoma, but age, stage, histologic type and grade were not significant for survival. However, the data of this study suggest that either a high LI or a high AI is associated with poor outcome in the small tumor only. Increased apoptosis is associated with tumor proliferation, but it did not predict clinical outcome. Although none of the proliferation measurement had independent prognostic significant when the effect of initial tumor size was taken into account. They suggested that LI and AI may be useful in discriminating outcome for patients with small tomors when managed by RT.

Crompton et al,[70] studied radiation-induce apoptosis in CD4 and CD8 T-lymphocytes from various cohorts of blood donors: healthy donors, cancer patients, cancer hypersensitive to RT, and ataxia telangiectasia patients who are highly sensitive to ionizing radiation. Blood samples were irradiated with 0-, 2-, or 9- Gy X-ray and incubated for 48 h after detection via labelling with FITC-conjugated antibodies that specific with CD4 and CD8 T-lymphocytes and then analyzed by the flow cytometer. Apoptosis was confirmed by light microscopy, electron microscopy, and by the use of apoptotic detection kit. Their

results demonstrated that the apoptotic response of T-lymphocytes from hypersensitive patients is significantly different to the response of T-lymphocytes from normal individuals. The radiation-induced apoptosis response of the CD4 and CD8 T-lymphocytes from both groups of hypersensitivity patients is significantly lower than the response of the CD4 and CD8 T-lymphocytes from normal individuals. They concluded that the leukocyte apoptosis assay appears to be a useful predictor of individuals likely to display increased toxicity to radiation therapy. However, validation of this requires a prospective study.

Birnhoim et al,[6] studied white blood cells from human volunteers. Bloods were irradiated with low dose (0.04-1.6 Gy/min.) of ^{60}Co γ- rays. Radiation-induced strand break are responsible for the increased rate of DNA unwinding that can be used as a sensitive measure of strand break. They detected for DNA strand break in white blood cells by FADU assay. Their results showed that 1 Gy of γ-irradiation produced a really observable effect upon unwinding rate and they suggested that FADU assay is simple, rapid, and sensitive, and it may have application in several areas related to human health.

Boreham et al,[5] tested the possibility of using apoptosis in human lymphocytes as a short-term biological dosimeter. They used blood samples from healthy male volunteers. The blood samples were irradiated at 37 $^{\circ}$C with 0 to 1.0 Gy and maintained in the culture for up to 96 hours post irradiation. Samples were harvested at various times and detected apoptotic cells by TdT and FADU assay. Their results showed that the dose dependent induction of apoptosis was significant after 48 h of incubation and apoptosis increased for up to 72 hours post-irradiation. The TdT assay and FADU assay seemed to give similar results when used to measure apoptosis at 1.0 Gy. Under their experimental condition, apoptosis in lymphocytes required many hours to develop. Apoptosis nuclei (TdT assay) and fragmented DNA (FADU assay) could be detected 24 hours post-irradiation and for 0.5 Gy and 1.0Gy reached a maximum between 48 and 72 hours. Apoptosis can be induced at low dose of 0.05 Gy, which is sensitive compared to other traditional cytogenetic endpoints. They suggest that radiation-induced apoptosis may be useful in assessing

individual sensitivity to radiation and apoptosis has good potential as a short-term accident biological dosimeter to present an overall cellular radiation sensitivity.

The process of apoptosis in many cell types is short-lived, but lymphocytes in cell culture display an arrested apoptosis after radiation exposure. The cells remain in this state for many days. Lymphocyte is easy to be collect from the body more than the specimen excised from tumor tissue and easy to detect apoptotic cells in laboratory. Thus, we study apoptosis in lymphocytes which represent other cells in the body, and detect apoptotic cells by TdT assay which is sensitive, and rapid.

CHAPTER III
MATERIALS AND METHODS

1. Specimens

twenty ml of Heparinized whole blood per donor were collected from 75 cervical cancer patients of the stage I - III with signed patients' informed consent. The normal controls are 15 healthy women who were blood donors at the National Blood Bank Thai Red Cross Society.

2. Materials

2.1 Aerosol resistance pipette tip : 200 μl. (Molecular Bio-Products, USA)

2.2 Autoclave tape (3M, USA)

2.3 Aluminum foil (Diamond®, USA)

2.4 Beakers : 50 ml, 1,000 ml. (Pyrex ®, USA)

2.5 Coplin staining jars

2.6 Culture flasks (Nunc , USA)

2.7 Cylinders (Pyrex ®, England)

2.8 Disposable gloves

2.9 Glass pipettes : 1 ml, 5 ml, 10 ml. (Witeg, Germany)

2.10 Heparinized Vacutainer 10 ml (Vacutainer®,USA)

2.11 Humidified chamber

2.12 Microscope glass cover slips (Chance, England)

2.13 Needle (Vacutainer System PrecisionGlide™, UK)

2.14 Plastic cover slips (ApopTag®)

2.15 Pasteur pipettes

2.16 Parafilm (American National Can™, USA)

2.17 Reagent bottles : 250 ml, 1000 ml (Duran®, Germany)

2.18 Slide film (Eritchome 400, Kodak)

2.19 Sterile polypropylene centrifuge tube : 15 ml., 50 ml. (Nunc™, USA)

2.20 Slide (Super Frost, Germany)

2.21 Slide box

2.22 Sterile membrane filters (Whatman®, Japan)

2.23 Slotted microscope slide staining dish

2.24 Tube rack

2.25 T 25 Tissue Culture flasks (Nunc™ , USA)

3. Equipment

3.1 Autoclave (HICLAVE™, HIRAYAMA)

3.2 CO_2-Incubator (REVCO ULTIMA)

3.3 Co-60 Teletherapy machines (Eldorado-78, Canada), Radiotherapy Unit, Department of Radiology, Faculty of Medicine, Chulalongkorn University.

3.4 Dark room

3.5 Differential counter

3.6 Fluorescence microscope (Olympus), Andrology Unit, Department of Obstertrics and Gynecology, Faculty of Medicine, Chulalongkorn University.

3.7 Freezer -20 °C

3.8 Fume hood (Model 252,NEWLAB®)

3.9 Hemocytometer (Boeco, Germany)

3.10 Incubator (Heraeus)

3.11 Bioharzard Lamina-flow hood (Gelman Science)

3.12 Light microscope (Olympus, Japan)

3.13 Low-speed centrifuge (Beckman)

3.14 Autopipette

3.15 pH meter (Eutech Cybernetics)

3.16 Refrigerator 4 °C, -20 °C (SANYO)

3.17 Timer

4. Reagents

4.1 General reagent

4.1.1 Absolute ethanol (Merk, Germany)

4.1.2 Acetic acid (Merk, Germany)

4.1.3 Fetal Bovine Serum (Gibco BRT,Germany)

4.1.4 Formaldehyde

4.1.5 Gentamycin (GOH)

4.1.6 Hank Buffered Salt Solution (HBSS) Powder (Gibco BRL)

4.1.7 Histopague®-1077 (Sigma, USA)

4.1.8 Heparin (LEO)

4.1.9 Hoeschts 33258 Dye solution

4.1.10 Hydrochloric acid : (Merk, Germany)

4.1.11 L-Glutamine (Gibco BRL)

4.1.12 Paraformaldehyde powder (Sigma, Germany)

4.1.13 Potassium chloride

4.1.14 Potassium hydrogen phosphate

4.1.15 RPMI 1640 (Gibco BRL)

4.1.16 Sodium chloride

4.1.17 Sodium hydroxide (Merk)

4.1.18 di- Sodium hydrogen phosphate monobasic

4.1.19 Sodium bicarbonate

4.1.20 Clorox

4.2 Reagent Kit

4.2.1 Apoptag®Fluorescein kits (Intergen)

4.3 Enzyme

4.3.1 Terminal deoxynucleotidyl transferase (TdT) Enzyme (Intergen)

5. Methods

- **Sampling of blood samples**

The blood samples used in this study were collected from cervical cancer patients receiving radiation therapy at King Chulalongkorn Memorial Hospital during 1999-2000 and from healthy women who are blood donor at The Nation Blood Bank, Thai Red Cross Society. The criteria for selection the sample is :

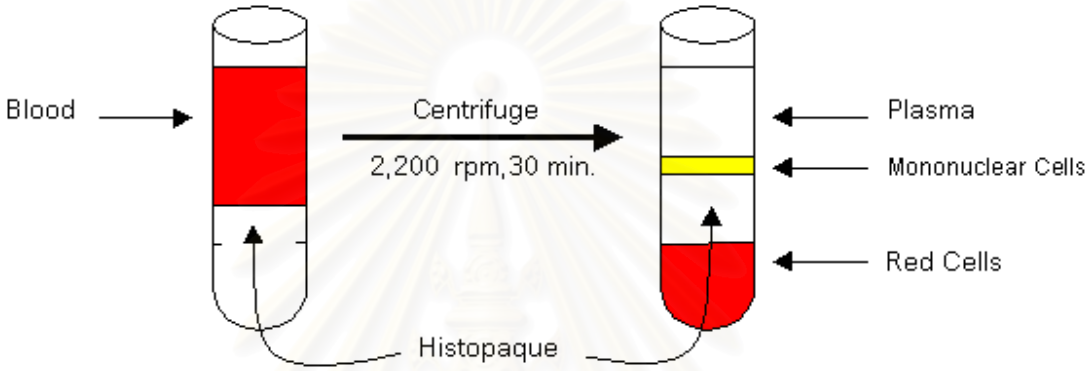
Patient group : Cervical cancer patients stage I- III, age 20-75 years, without other disease. The patients were treated with a combination of external bream therapy and high-dose rate intracavitary irradiation. External whole pelvis irradiation was performed with anteroposterior and posteroanterior parallel opposed portals. The dose were 2 Gy per fraction, and treatment was give five time per week; total doses averaged 75 Gy per patient.

Control group : Thai healthy women age 20-75 years, neither smoke nor alcohol drinking, and do not taking any drug 14 days before the blood donation.

- **Lymphocytes Isolation**

1. Pipette 5 ml Histopaque-1077 at room temperature into each 15 ml polypropylene centrifugue tubes, the total of 6 tubes.
2. Pool the blood collected into one 50 ml centrifugue tubes.
3. Mix the blood 1:1 with Hank Buffered Salt Solution (HBSS) add 2 μl Heparin at room temperature.
4. Transfer 9 ml of the blood / HBSS mixture on top of the histopaque in each tube. Be careful not to mix the two parts together, and cap the tube tightly.
5. Centrifuge at 2,200 rpm for 30 minutes at room temperature.

6. Using a pipette to remove the top plasma layer from each tube. Discard this layer into a container of Clorox.
7. Use a swirling motion with the pipette, collect the next layer (white ring of lymphocytes) from each tube. The next layer is the residual histopaque. Try not to collect much of this layer and do not collect the red cell layer which sits at the bottom of the tube. (See attached diagram)



8. Immediately transfer each lymphocytes ring to a new, sterilize, polypropylene 15 ml centrifuge tube. Add 12.5 ml HBSS (+ 2 μl/ml Heparin + 1 % Fetal Bovine Serum) to each tube. Cap tightly, then invert the tubes 3 time to mix.
9. Centrifuge 1,000 rpm for 10 minutes at room temperature.
10. Decant the supernatant from each centrifuge tube into a Clorox container. Flip to softly disperse the lymphocyte pellet. Add 5 ml HBSS (+ 2 μl/ml Heparin + 1 % Fetal Bovine Serum) to the first tube. The mixture of HBSS and cells were withdrawed up and down 3 times to further dispersed the cells. Transfer the cell suspension from first tube to a second centrifuge tube and repeat the mixing process. Continue the procedure until all of the cells are in the last tube.
11. Add an additional 5 ml HBSS / Heparin / 1 % Fetal Bovine Serum to this last tube to bring the volume to 10 ml. Mix by pipetting up and down 3 times.
12. Centrifuge 1,000 rpm for 10 minutes at room temperature.

13. Decant the supernatant into the Clorox container and mix the cells. Add complete RPMI medium (RPMI 1640 media + 5.2 % Fetal Bovine Serum + L-Glutamine + Gentamycin) to 10 ml. Mix by pipetting up and down 3 times.
14. Put an aliquot cell suspension on the counting chamber and count the cells. Calculate the total cell counts and add complete RPMI medium to obtain the suspension of cells at the concentration of $4x10^5$ cells/ml for culture. Transfer 10 ml of this cell suspension to 4 culture flasks.
15. Separate culture flasks into 2 groups: the control and the irradiation group which is irradiated at 0.5 Gy of Cobalt-gamma ray.
16. Incubate all the culture flasks in CO_2 incubator at 37 ^{o}C, 97% humidity, 0.35% CO_2. The cell will be harvested at 24 and 48 hours.

- **Fixing Cells for ApopTag.**

1. Mix cells in the culture flask by pipetting up and down 3 times to resuspend the cells more evenly.
2. Transfer 2.0 ml of the $4x10^5$ cells /ml into the new polyporpylene centrifuge tube. (800,000 cells)
3. Centrifuge 1,000 rpm for 10 minutes at 4 ^{o}C.
4. Aspirate out the supernatant and flip to softly disperse the lymphocyte pellet.
5. Add 2 ml PBS at 4 ^{o}C to each tube. Mix cell by pipetting up and down 3 times.
6. Centrifuge 1,000 rpm for 10 minutes at 4 ^{o}C.
7. Aspirate out the supernatant and flip to softly disperse the lymphocyte pellet.
8. Add 2 ml PBS at 4 ^{o}C to each tube. Mix cell by pipetting up and down 3 times
9. Centrifuge 1,000 rpm for 10 minutes at 4 ^{o}C.
10. Aspirate out the supernatant and flip to softly disperse the lymphocyte pellet.
11. Resuspend the cells in 250 μl PBS/1% Paraformaldehyde fixative. Let sit for at least 10 minutes at room temperature and proceed to the slidemaking protocol. For storing, add PBS/1% Paraformaldehyde fixative to 10 ml volume and store in the refrigerator until use.

- **ApopTag Slidemaking Protocol**

1. Clean microscope slides by rinsing with double distilled, deionized water and dry.
2. Centrifuge the paraformaldehyde fixed sample 1,000 rpm for 10 minutes at room temperature.
3. Carefully aspirate the supernatant out without disturbing cell pellets. Flip to softly disperse the lymphocyte pellets.
4. Add 100 μl PBS / 1% Paraformaldehyde fix to resuspend cells. Flip to softly disperse the lymphocyte pellets.
5. Drop 20 μl of this cell suspension onto a clean slide.
6. Allow the slides to air dry for 1 hour in a fume hood or laminar flow carbinet.
7. Fix by placing the slides in absolute ethanol overnight at –20 ^{o}C.
8. Transfer slides from Absolute ethanol. Allow slides to air dry for 1 hour in a fume hood or laminar flow cabinet, and mark around the area of the specimen.
9. Wash slides for 5 minutes in PBS at room temperature for 3 times.
10. Allow slides to air dry, put one drop (13.5 μl) of Equilibration buffer (ApopTag Kit) to the area marked on the slide. Cover the marked area with plastic coverslip (Figure 13) and incubate at room temperature for 5 minutes on bench top at room temperature.
11. After incubation, carefully remove the coverslip and dry around the marker area with absorbent. (Note : The positive control slide was treated with DNase I, and incubated in CO_2 incubator 1 hour before going to step 12.)
12. Add 13.2 μl of TdT mixture prepared freshly or within 6 hours to the specimen area. Cover with plastic coverslip and incubate in CO_2 incubator at 37 ^{o}C, 97% humidity, 0.35% CO_2 for 1 hour. (Note : The negative control slide was created by omitting TdT enzyme.)

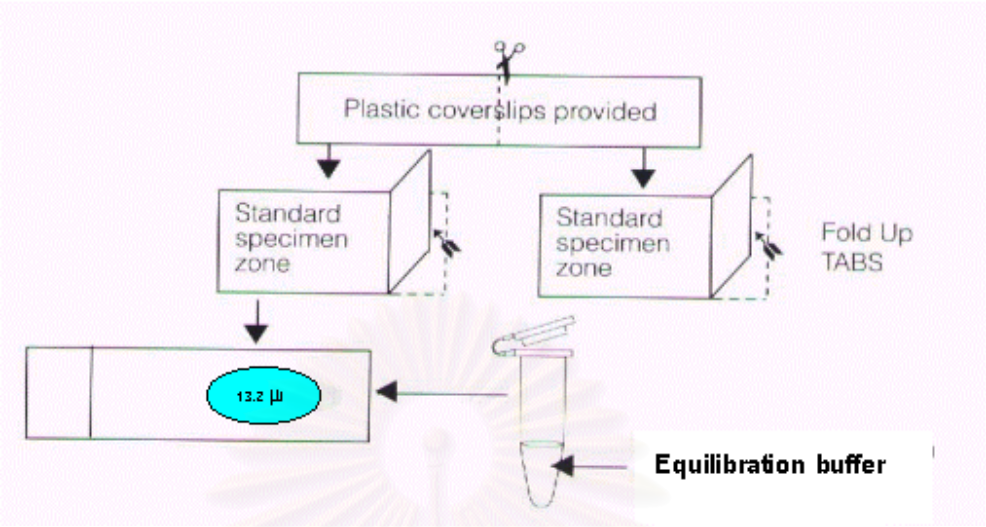


Figure 13 Demonstrate the slide marking and cover with plastic coverslip.[12]

13. After incubation. Carefully remove the coverslip and dry off around the marker area with absorbent.
14. Place the slides into the Coplin staining jar containing a warm stop wash buffer, incubate at 37 ^{o}C for 30 minutes.
15. Wash slides for 5 minutes in PBS at room temperature 3 times.
16. Allow the slides to air dry in a fume hood or laminar flow cabinet.
17. Add 13.2 µl FITC mixture prepared freshly or within 6 hours onto the specimen area. Cover with plastic coverslip, incubate 45 minutes in humidified chamber place in a dark room at room temperature. (Note ; Avoid exposure to light as much as possible)
18. After incubation, carefully remove coverslip and dry off around the sample area. Wash slides 3 times for 5 minutes in PBS in the dark room.
19. Rinse the slides 1 minute with Hoechst dry solution, then rinse the slides in PBS for 1 minute in the dark room.
20. Allow the slides to air dry in a fume hood or laminar flow carbinet. Add 10 µl DAPI-Antifade solution to the specimen area and cover with a standard glass coverslip and mount. Develop the slides in the dark room for 10 minutes before viewing by fluorescence microscope. If storage of the slides are

required, put some rubber cement to edges of the coverslip and store at -20 ^{o}C in a dark box.

21. The slides were viewed under a fluorescence microscope for monitoring apoptotic cells. The lymphocyte cells were determined by counting the total lymphocytes more 1000 cells per slide and counted the apoptotic cells in the same field under the blue filter. The apoptotic index and apoptotic activity were analyzed.

- **Statistical analysis**

1. The comparison between the apoptotic activity of lymphocyte taken from patient at various stage of cervical cancer and healthy women was determined by unpaired T test, and the comparison of the apoptotic activity of the patients at various time interval of treatment was determined by one way Analysis of Variance (ANOVA) from the Statistical Packages for the Social (SPSS) program. [71,72]
2. The quality control for intra assay to detect apoptotic cells was done by counting the same slide 10 time within a day. The Optimum Condition of Variance (OCV) was calculated and should not exceed 10 %. [73] The calculation formular is shown below :

$$\% CV = SD/\bar{X} \times 100$$

$$SD = \sqrt{\Sigma(X_i - \bar{X})^2 / n-1}$$

$$\bar{X} = \Sigma X_i / n$$

CHAPTER IV
RESULTS

Patients and control group

The cervical cancer patients were treated with radiation therapy (RT). Two patients were treated with a combination of surgery and high dose rate intracavitary irradiation. The mean age of the patients was of age 53.3 years (range 29 - 72 years). The mean age of control group (healthy women) was 39.8 years (range 21 - 59 years).

Detection of apoptotic cells

The apoptotic cells were viewed under a fluorescence microscope with 200 - 1000 X of magnification. All the cells in each microscopic field gave blue fluorescent appearance (Figure 16A) when first viewed without any filter. Then, the same field had to be examined again through a blue filter. The apoptotic cells would appear as light green circles (Figure 16B). A positive control slide (Figure 14A & B) was prepared by treating the lymphocytes with DNase I to yield DNA fragmentation. A negative control slide shown in Figure 15A & B was prepared simultaneously from the lymphocytes by omitting TdT enzyme from the ApopTag slidemaking protocol. Both the positive control and negative control must be include in each test. A quality control check was performed once a month. The mean of % OCV was 5.21 % (range = 4.27 % - 8.97 %).

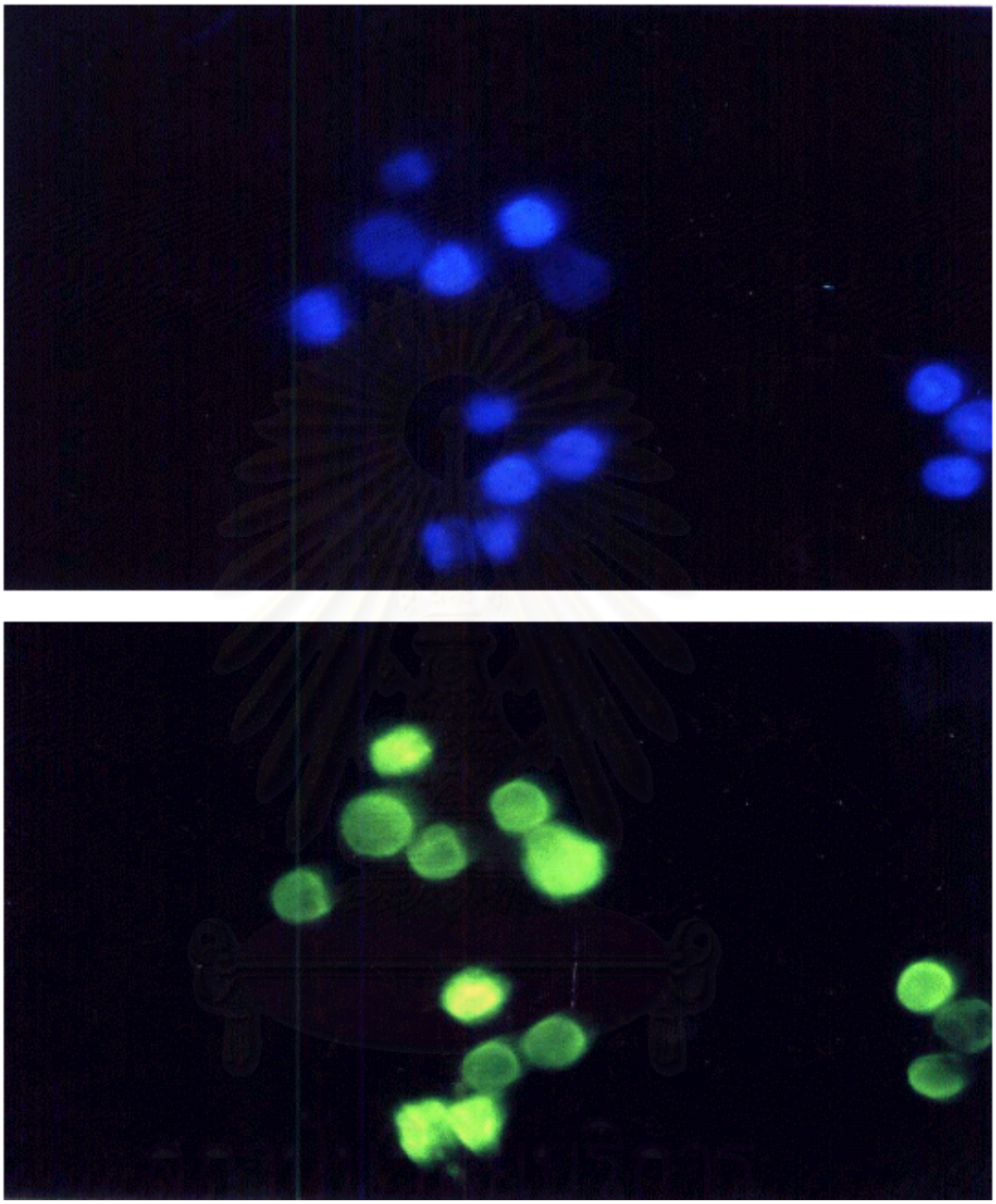


Figure 14 The positive control slide was prepared from lymphocytes exposed *in vitro* to 0.5 Gy Co irradiation , then, detected the apoptotic cells were detected by TdT assay. The positive control was prepared from lympphocytes treated with DNase I to induce DNA fragmentation. (A) Without the blue filter, all the cells are shown as the blue cells at 1000X. (B) Under the blue filter, the apoptotic cells are seen as the light green cells in the same field at 1000X. In this field, all lymphocytes are apoptotic cells.

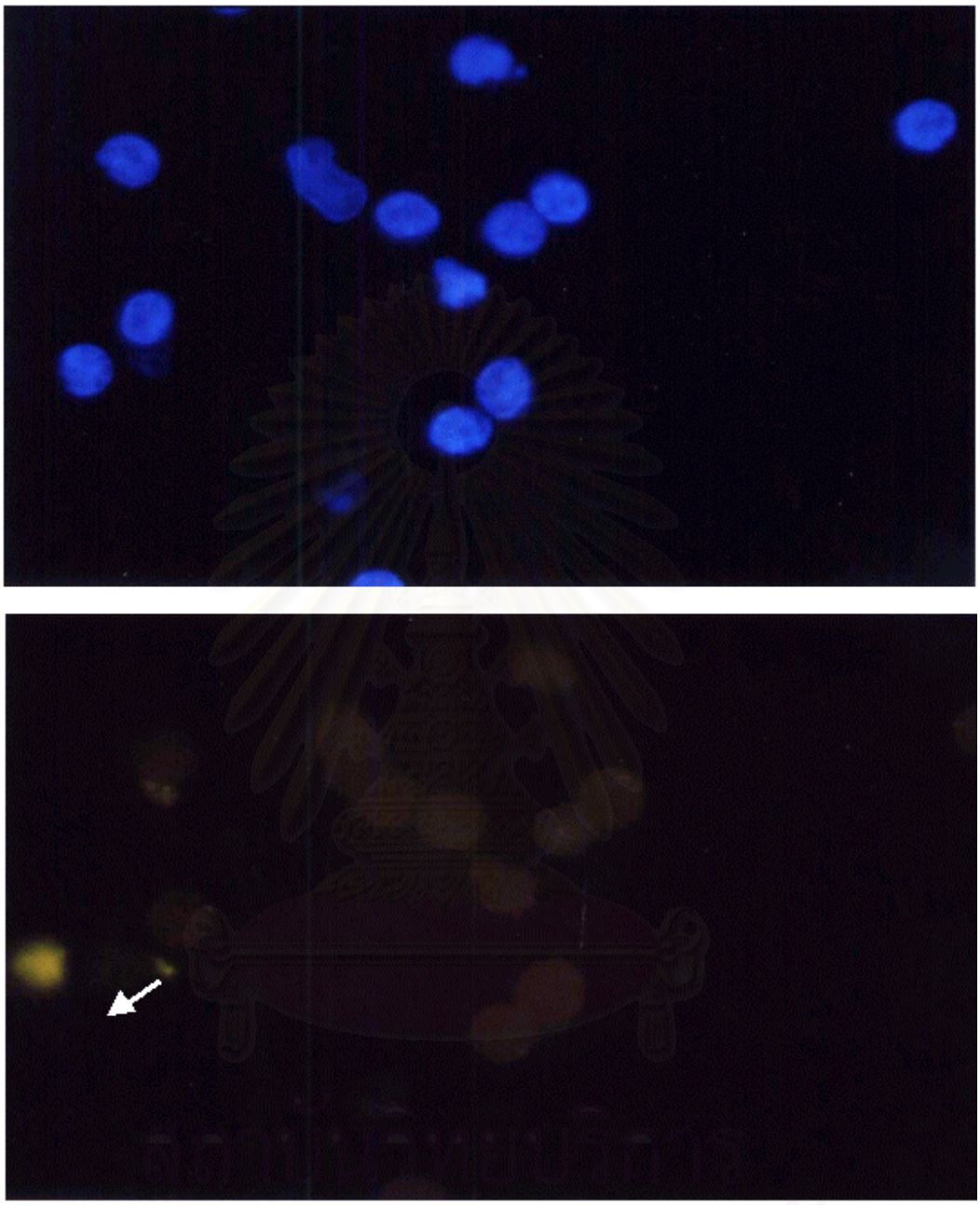


Figure 15 The negative control slide was prepared from lymphocytes exposed *in vitro* to 0.5 Gy Co irradiation, then, the apoptotic cells were detected by TdT assay. The negative control slide was obtained by omitting the TdT enzyme from the labeling mix in the ApopTag slidemaking protocol. (A) Without the blue filter, all the cells are seen as the blue cells at 1000X. (B) Using the blue filter, no apoptotic cells can be observed in the same field at 1000X. The arrow head is the residual of fluorescent color.

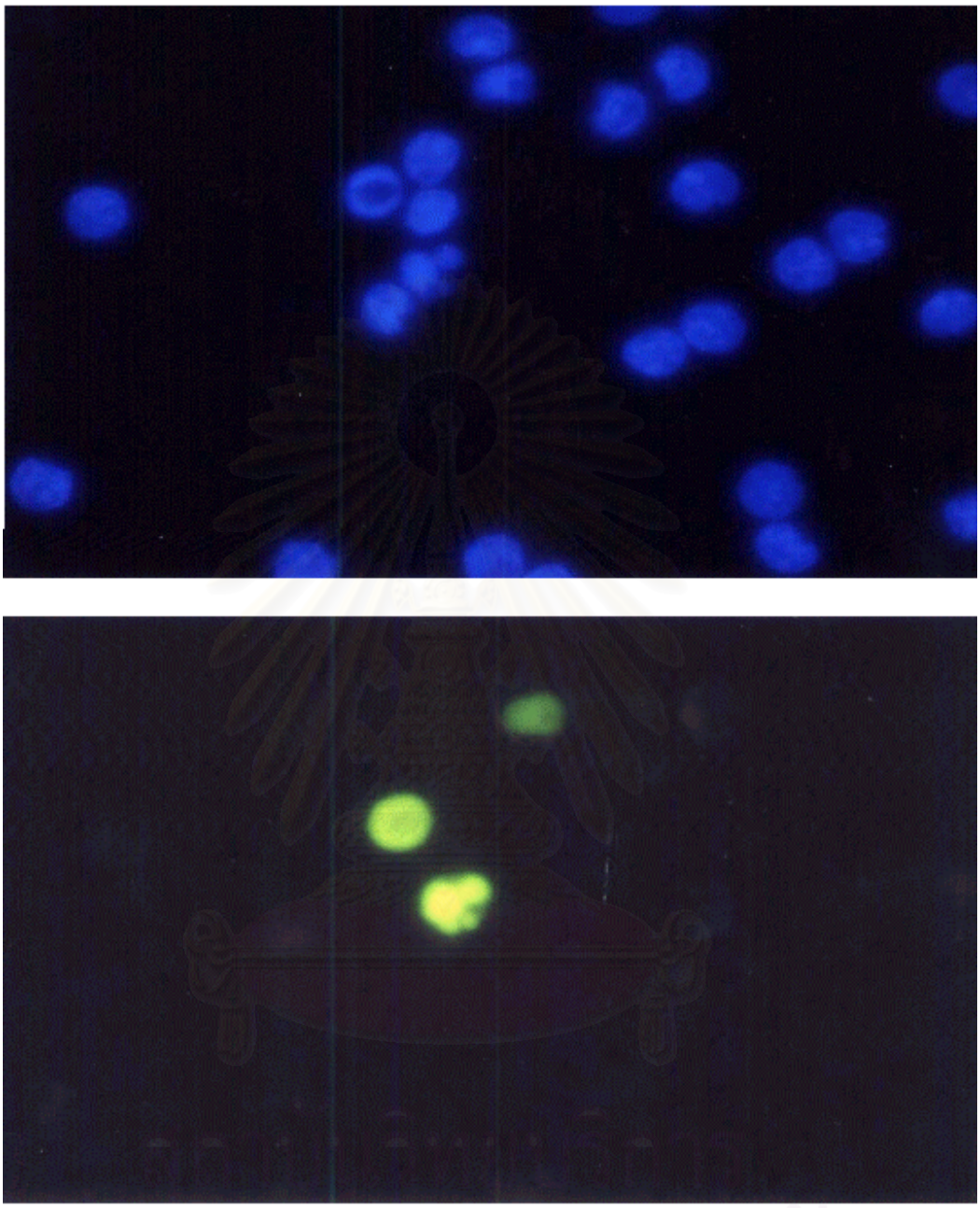


Figure 16 The picture of total and apoptotic cells from one of studied sample. The lymphocytes were exposed *in vitro* to 0.5 Gy Co irradiation, then, the apoptotic cells were detected by TdT assay. (A) Without the blue filter, all the cells are seen as the blue cells at 1000X. (B) Through the blue filter, the apoptotic cells are seen as the light green cells within the same field at 1000X. The arrows indicate the apoptotic cells in which the apoptotic bodies can be seen.

Statistical analysis

The detailed data on statistical analysis were presented in Appendix B.

Percent apoptotic activity in cervical cancer patients

I. Cervical cancer, Stage I

The percent apoptotic activity of stage I cervical cancer patients had been detected in the lymphocytes at 24 hours after the induction by low dose radiation. The results indicated that the percent apoptotic activity of pretreated patients was lower than control group without any statistical significance (p=0.116; Appendix B). When compare the percent apoptotic activity of pretreated patients to the posttreated patients at various time intervals, the data showed an increase at 1 and 3 month, then slightly dropped at 1 year (Table 4). The percent apoptotic activity of the pretreated group was significantly lower than the 5 year posttreated group (p=0.032). There was no significant difference between the 5 year posttreated group and the control group (p=0.713), (Table 4; Figure 17A). The results of percent apoptotic activity in lymphocytes at 48 hours (Table4; Figure 17B) after the induction by low dose radiation demonstrated a similar pattern as the above 24 hours data. Statistical analysis also exhibited the same pattern (Appendix B).

The spontaneous apoptosis in lymphocytes that occurred during the isolation and culture process was showed in Appendix C. The spontaneous apoptosis in pretreated group was lower than the control group. After radiation therapy, there was a slight increased in spontaneous apoptotic index in all the posttreated groups.

Figure 18 gave the presentation of the mean $\pm$ SD of percent apoptotic activity in lymphocytes taken from stage I, cervical cancer patients at various time intervals; matched age and sex blood donors as controls in the bar graphic features. The percent apoptotic ctivity of lymphocytes for the 24 hours and 48 hours after the induction by low dose irradiation was compared.

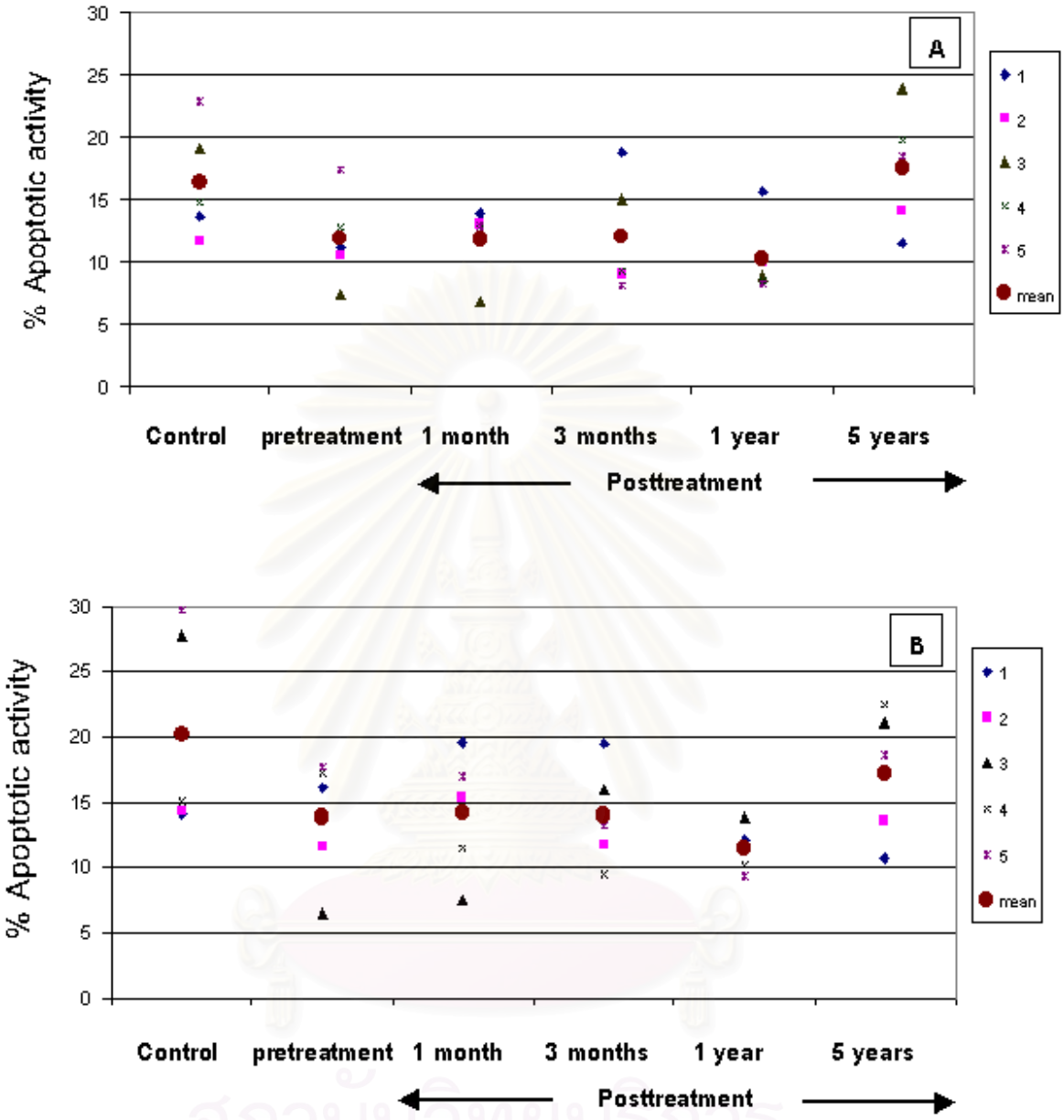


Figure 17 Comparison of the percent apoptotic activity in lymphocytes taken from the controls, healthy women and patients with stage I cervical cancer at various time intervals. The apoptotic cells were detected by TdT assay at 24 hours (A) and 48 hours (B) after low dose irradiation.

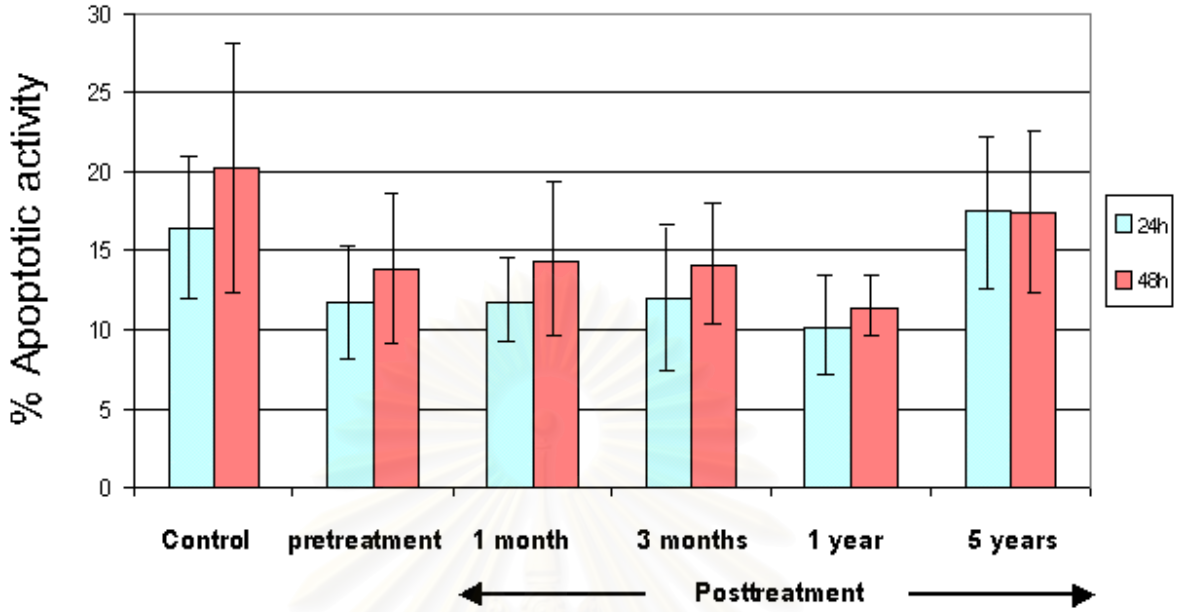


Figure 18 Comparison of the mean ± SD of percent apoptotic activity in lymphocytes obtained from the controls, healthy women to patients with stage I cervical cancer at various time intervals. The apoptotic cells were detected by TdT assay at 24 hours and 48 hours after low dose irradiation.

Table 4 Percent apoptotic activity data found in lymphocytes taken from stage I cervical cancer patients at various time intervals:pretreatment; 1 month, 3 months, 1 year and 5 years after the completion of radiation therapy. The apoptotic cells were detected by TdT assay at 24 hours and 48 hours after low dose irradiation.

Subjects	Control group		Pretreatment		Posttreatment							
					1 month		3 months		1 year		5 years	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	13.60	14.10	11.10	16.10	13.90	19.60	18.80	19.45	15.60	12.10	11.50	10.70
2	11.65	14.35	10.50	11.60	13.00	15.40	8.95	11.80	9.95	11.60	14.10	13.65
3	19.15	27.80	7.40	6.50	6.70	7.50	15.05	16.00	8.85	13.90	23.85	21.10
4	14.80	15.10	12.80	17.30	12.60	11.55	9.25	9.55	8.50	10.25	19.75	22.45
5	22.85	29.80	17.35	17.80	12.85	17.05	8.10	13.30	8.20	9.40	18.50	18.65
Mean	16.41	20.23	11.83	13.86	11.81	14.22	12.03	14.02	10.22	11.45	17.54	17.31
SD	4.53	7.86	3.65	4.79	2.90	4.76	4.68	3.84	3.08	1.74	4.85	4.99

The data showed in Figure 19 was the percent apoptotic activity of lymphocytes of two patients with Stage I, cervical cancer. One patient gave a very good cooperation since she volunteered to enter our study at the beginning until 1 year after the completion of radiation therapy. Another patient came at 1 and 3 months after radiation treatment. The percent apoptotic activity at 24 and 48 hours after low dose irradiation was presented in Figure 19A, and 19B respectively.

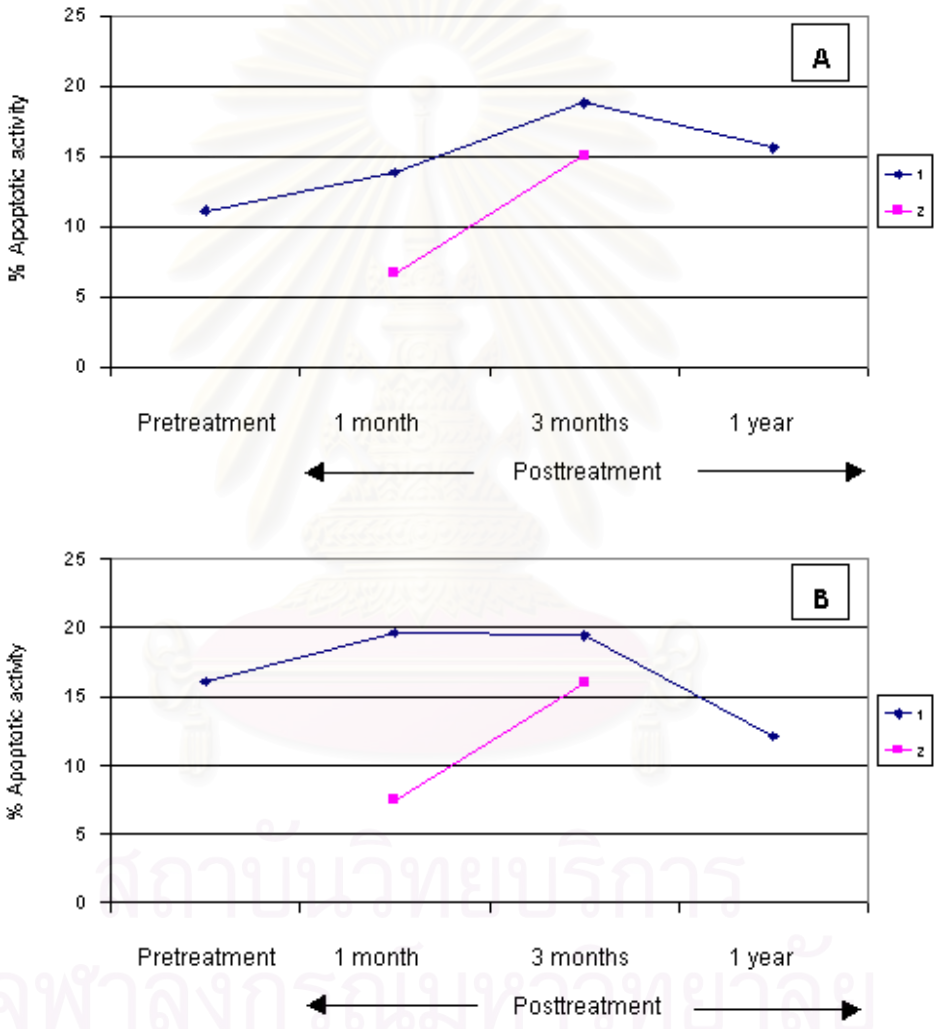


Figure 19 The percent apoptotic activity in lymphocytes from two patients with Stage I cervical cancer. These two patients had attended to the clinic during the study period. The apoptotic cells were detected by TdT assay at 24 hours (A) and 48 hours (B) after low dose irradiation.

II. Cervical cancer, Stage II

The percent apoptotic activity from Stage II cervical cancer patients had been detected in lymphocytes at 24 hours (Table 5; Figure 20A) and 48 hours (Table 5; Figure 20B) after low dose irradiation. The change of percent apoptotic activity found in patients with Stage II, cervical cancer were similar to those data shown in Stage I. Comparison of the percent apoptotic activity in lymphocytes (48 hours after low dose irradiation) taken from the 5 years group to the pretreated group had demonstrated a statistical significant increase ($p=0.007$; Appendix B).

The spontaneous apoptosis in lymphocytes found in patients with stage II was similar to stage I. (Appendix C)

The bar graph in Figure 21 was a comparison of the mean $\pm$ SD of percent apoptotic activity in lymphocytes between the Stage II, cervical cancer patients and the matched age and sex blood donor controls. The data for both 24 hours and 48 hours percent apoptotic activity were shown.

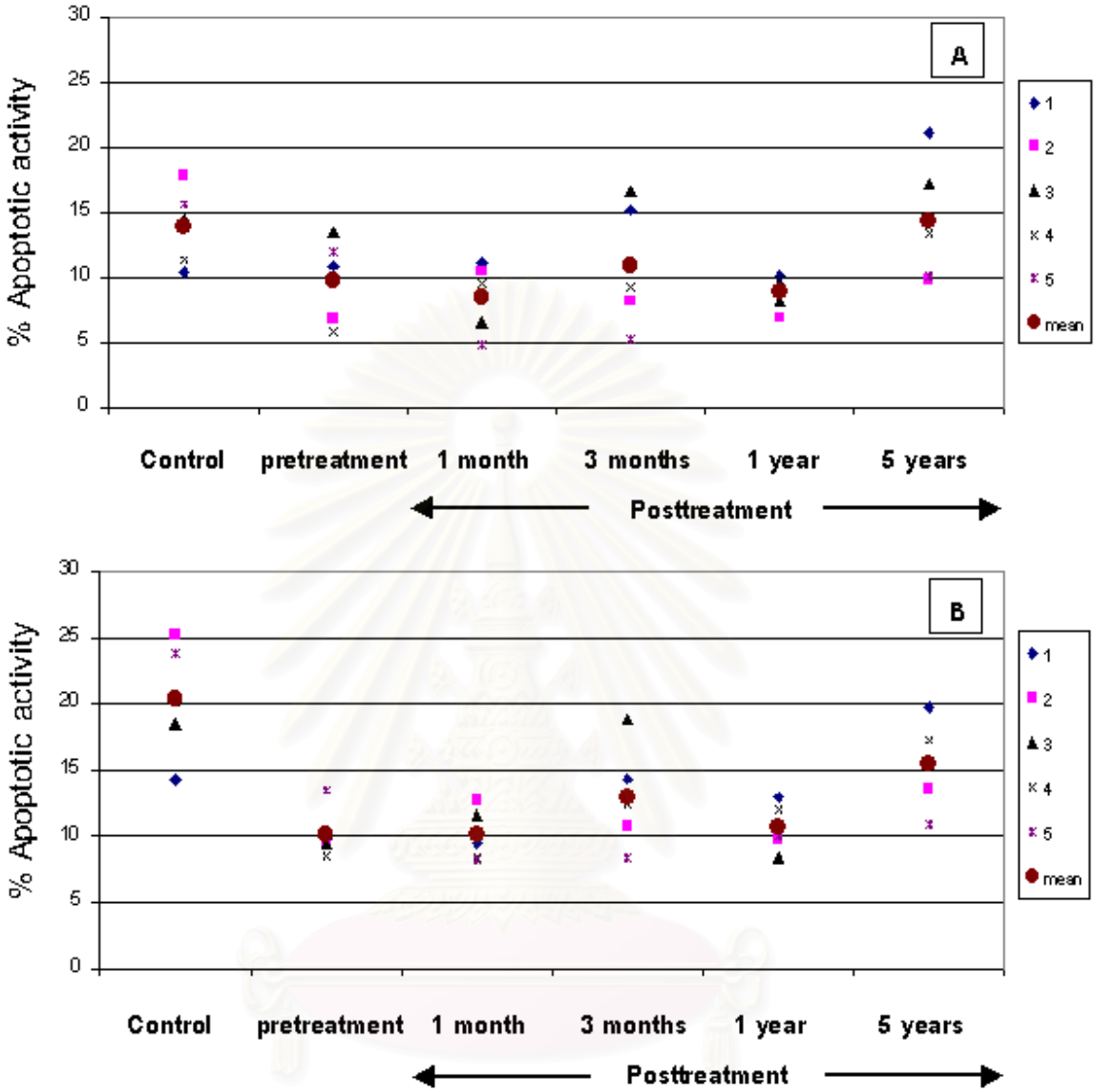


Figure 20 Comparison of the percent apoptotic activity in lymphocytes collected from the controls, healthy women and patients with stage II cervical cancer at various time intervals. The apoptotic cells were detected by TdT assay at 24 hours (A) and 48 hours (B) after low dose irradiation.

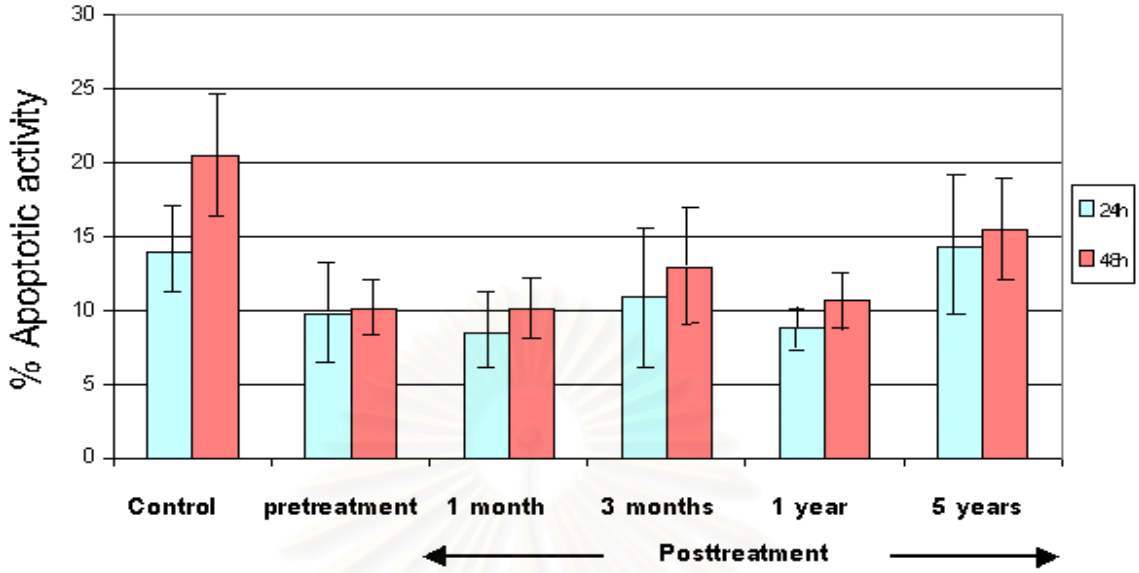


Figure 21 Comparison of the mean ± SD of percent apoptotic activity in lymphocytes obtained from the controls, healthy women to patients with stage II cervical cancer at various time intervals. The apoptotic cells were detected by TdT assay at 24 and 48 hours after low dose irradiation.

Table 5 Percent apoptotic activity data found in lymphocytes taken from stage II cervical cancer patients at various time intervals: pretreatment; 1 month, 3 months, 1year and 5 years after the completion of radiation therapy. The apoptotic cells were detected by TdT assay at 24 hours and 48 hours post-irradiation.

Subjects	Control group		Pretreatment		Posttreatment							
					1 month		3 months		1 year		5 years	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	10.40	14.30	10.90	9.85	11.10	9.50	15.20	14.35	10.15	12.95	21.10	19.80
2	17.90	25.25	6.80	9.50	10.50	12.70	8.20	10.75	6.90	9.85	9.85	13.55
3	14.40	18.50	13.50	9.50	6.60	11.65	16.65	18.85	8.20	8.40	17.20	15.60
4	11.35	20.20	5.80	8.50	9.60	8.40	9.30	12.40	9.80	12.05	13.40	17.30
5	15.65	23.85	11.95	13.50	4.80	8.20	5.25	8.40	9.20	10.05	10.10	10.95
Mean	13.94	20.42	9.79	10.17	8.52	10.09	10.92	12.95	8.85	10.66	14.33	15.44
SD	3.08	4.37	3.34	1.93	2.71	2.00	4.83	3.96	1.32	1.82	4.82	3.40

The percent apoptotic activity presented in Figure 22 (24 hours, A; 48 hours, B) was the data collected from 3 patients with Stage II, cervical cancer. The percent apoptotic activity in lymphocytes from patients 1 and 3 was collected at pretreatment and 3 months after completion of radiation therapy. The data for patient 2 were collected at pretreatment and 1 month after finishing radiation therapy. The percent apoptotic activity in all 3 patients was increased after finishing radiation treatment regimen.

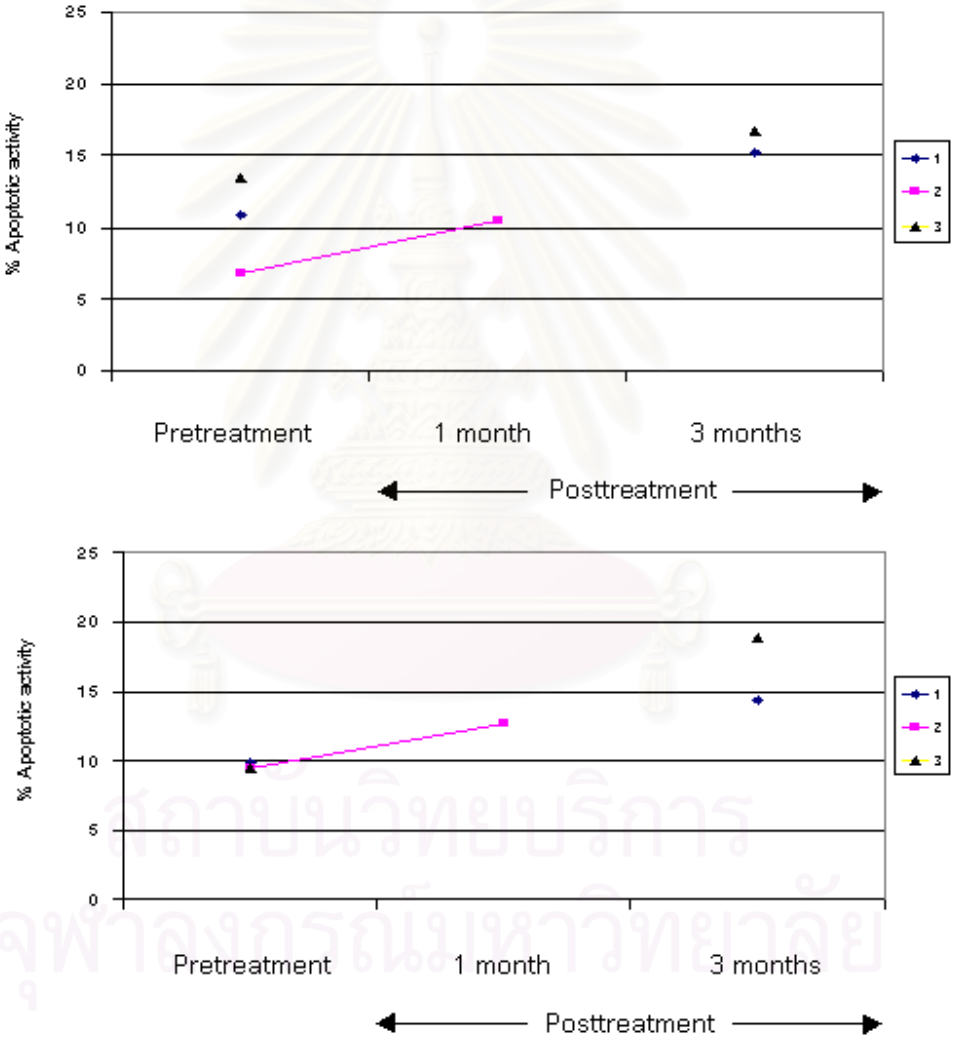


Figure 22 The percent apoptotic activity in lymphocytes taken from three patients with stage II cervical cancer. These three patients had attended to the clinic at different interval during this study. The apoptotic cells were detected by TdT assay at 24 hours (A) and 48 hours (B) after low dose irradiation.

III. Cervical cancer, Stage III

At 24 hours after low dose irradiation, the percent apoptotic activity in lymphocytes taken from Stage III, cervical cancer patients of the pretreatment group was significantly lower than the control group (p=0.024; Appendix B; Table 6 and Figure 23 A). It also showed significantly lower than the 5 years posttreatment group. (p=0.001)

Table 6 and Figure 23B were the results of the percent apoptotic activity in lymphocytes at 48 hours after low dose radiation induction. The result in pretreated patients of Stage III, cervical cancer was significantly lower than both the control (p=0.000; Appendix B) and the 5 years posttreatment group (p=0.000). The percent apoptotic activity in the 5 years posttreatment group was much lower than the control group with significance (p=0.015)

The spontaneous apoptosis in lymphocytes found in patients with stage III showed that the spontaneous apoptsis in the pretreated group was lower than the control group , and it was increased after radiation therapy. (Appendix C)

The summarized data taken from Table 6 and Figure 23A and B were presented as a bar graph in Figure 24.

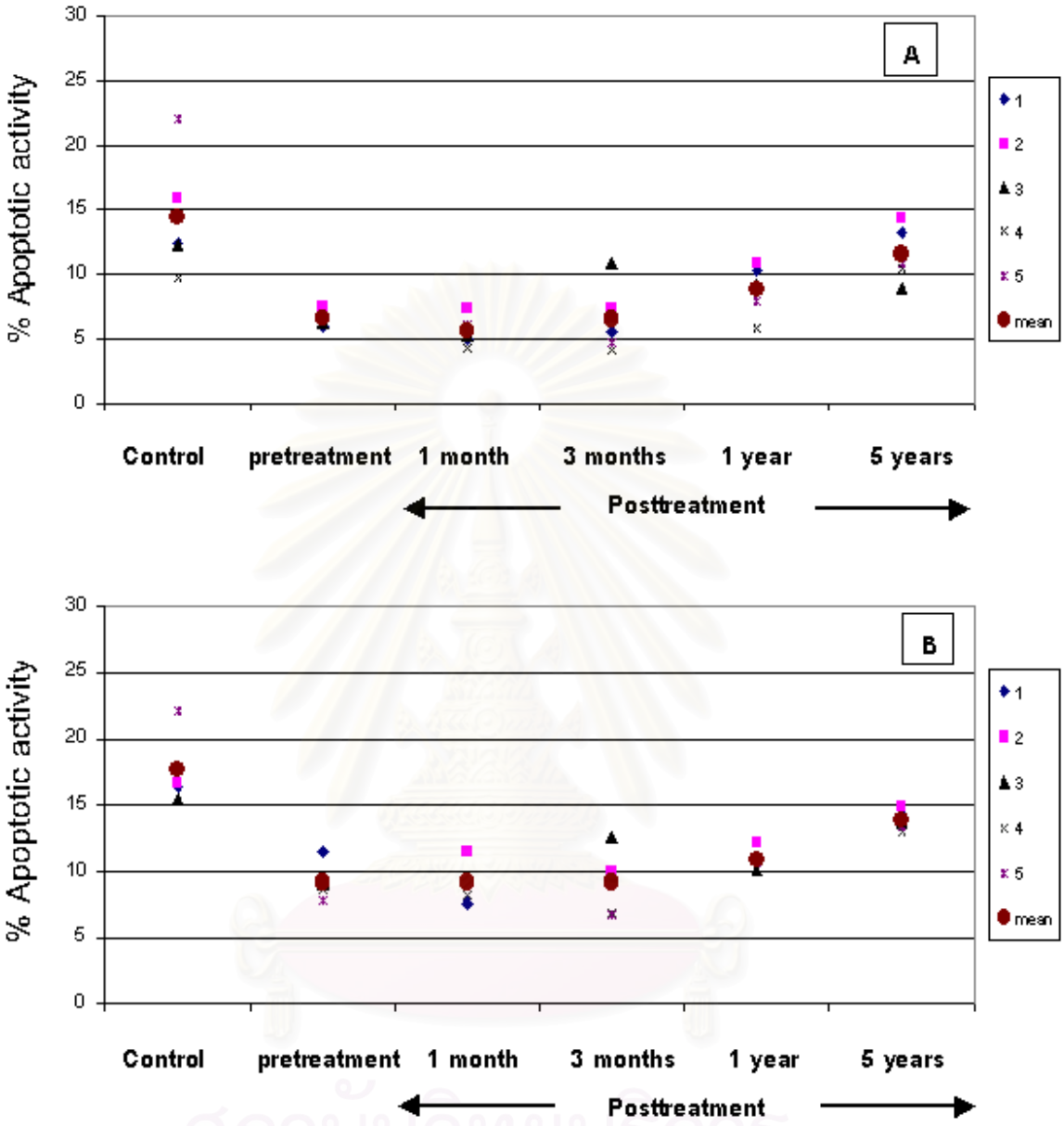


Figure 23 Comparison of the percent apoptotic activity in lymphocytes obtained from the controls, healthy women patients with stage III cervical cancer at various time intervals. The apoptotic cells were detected by TdT assay at 24 hours (A) and 48 hours (B) after low dose irradiation.

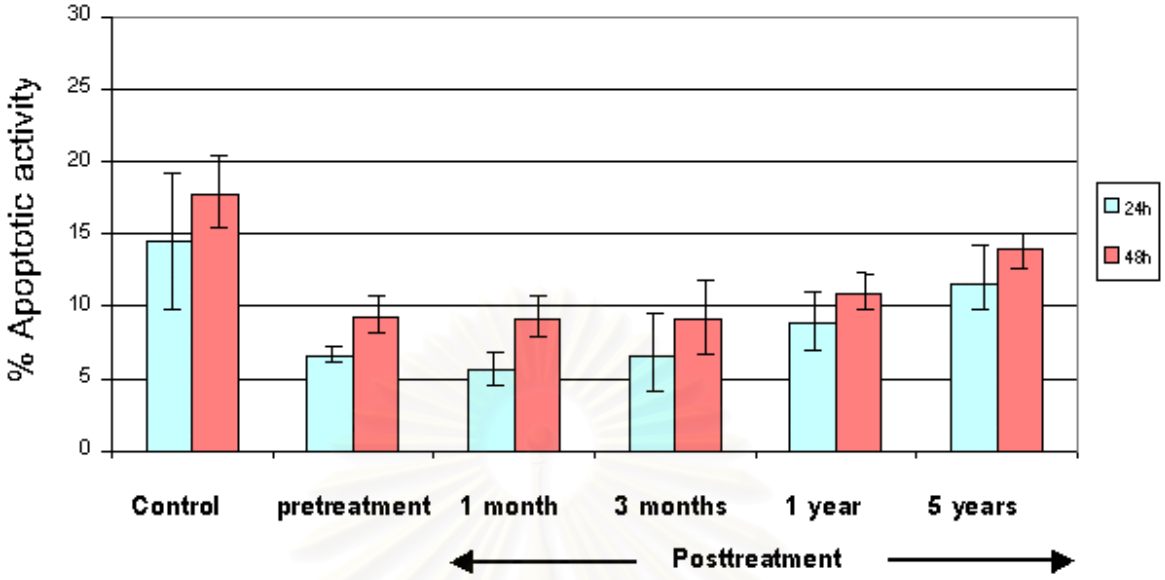


Figure 24 Comparison of the mean ± SD of percent apoptotic activity in lymphocytes collected from the controls, healthy women to patients with stage III cervical cancer at various time intervals. The apoptotic cells were detected by TdT assay at 24 hours and 48 hours after low dose irradiation.

Table 6 Percent apoptotic activity data found in lymphocytes taken from stage III, cervical cancer patients at various time intervals: pretreatment; 1 month, 3 months , 1year and 5 years after the completion of radiation therapy. The apoptotic cells were detected by TdT assay at 24 hours and 48 hours post-irradiation.

Subjects	Control group		Pretreatment		Posttreatment							
					1 month		3 months		1 year		5 years	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	12.35	16.45	5.95	11.50	5.00	7.60	5.50	9.65	10.30	10.65	13.20	14.40
2	15.85	16.65	7.50	9.00	7.40	11.50	7.40	9.95	10.80	12.20	14.40	14.95
3	12.2	15.40	6.20	9.10	5.20	9.35	10.85	12.55	9.20	10.10	8.95	13.65
4	9.8	17.75	6.70	8.60	4.25	8.25	4.15	6.85	5.85	10.80	10.40	13.00
5	22.05	22.15	6.60	7.80	6.05	9.10	4.75	6.75	7.85	10.70	10.80	13.45
Mean	14.45	17.68	6.59	9.20	5.58	9.16	6.53	9.15	8.80	10.89	11.55	13.89
SD	4.76	2.63	0.59	1.38	1.20	1.48	2.70	2.42	2.00	0.78	2.21	0.78

One patient with Stage III, cervical cancer had attended to this study at pretreatment and 1 month after the completion of radiation therapy. The percent apoptotic activity in lymphocytes at both 24 hours and 48 hours after low dose irradiation were slightly increased as shown in Figure 25.

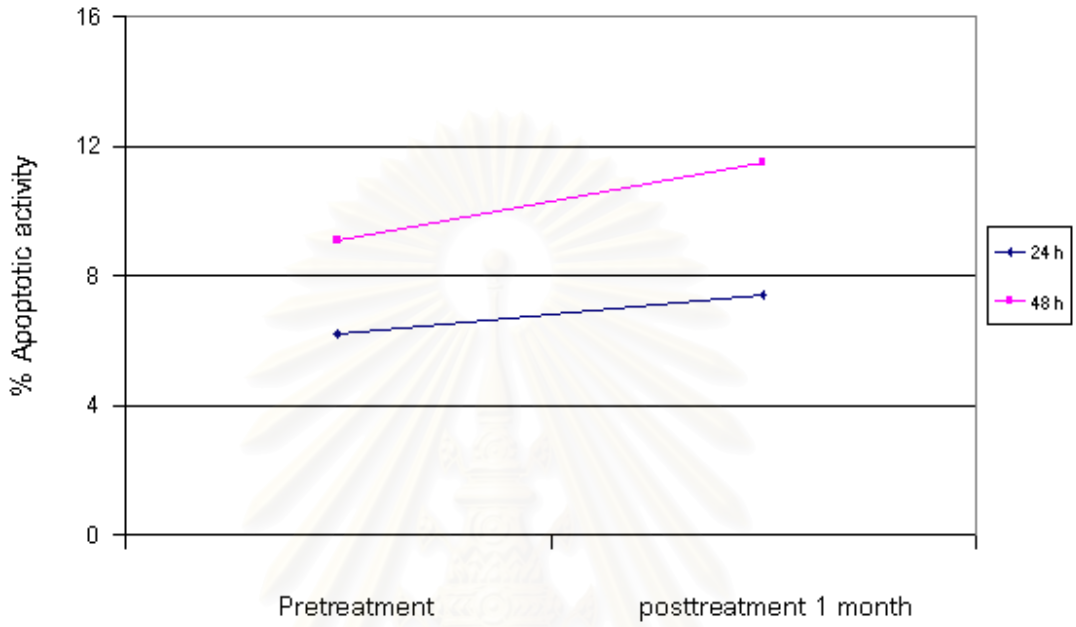


Figure 25 The percent apoptotic activity in lymphocytes taken from one patient with Stage III, cervical cancer. This patient had attended to the clinic at pretreatment and one month after completion of radiation therapy. The apoptotic cells were detected by TdT assay at 24 hours and 48 hours after low dose irradiation.

The mean of percent apoptotic activity in lymphocytes from each Stage of cervical cancer and its matched control had been compared. The data were presented in Table 7 and as a bar graph in Figure 26A, and 26B for the 24 hours and 48 hours respectively. The overall results demonstrated that the percent apoptotic activity in pretreatment cervical cancer patients were lower than the control group. After the completion of radiation therapy, the percent apoptotic activity in lymphocytes was gradually increased approaching to the control level.

Table 7. Comparison of the percent apoptotic activity in lymphocytes between Cervical Cancer Stage I; Stage II and Stage III at various time intervals. The apoptotic cells were detected by TdT assay at 24 and 48 hours post-irradiation. The data are expressed as mean and SD in the brackets.

Stage	Control group		Pretreatment		Posttreatment							
					1 month		3 months		1 year		5 years	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
I	16.41	20.23	11.83	13.86	11.81	14.22	12.03	14.02	10.22	11.45	17.54	17.31
	(4.53)	(7.86)	(3.65)	(4.79)	(2.90)	(4.76)	(4.68)	(3.84)	(3.08)	(1.74)	(4.85)	(4.99)
II	13.94	20.42	9.79	10.17	8.52	10.09	10.92	12.95	8.85	10.66	14.33	15.44
	(3.08)	(4.37)	(3.34)	(1.93)	(2.71)	(2.00)	(4.83)	(3.96)	(1.32)	(1.82)	(4.82)	(3.40)
III	14.45	17.68	6.59	9.20	5.58	9.16	6.53	9.15	8.80	10.89	11.55	13.89
	(4.76)	(2.63)	(0.59)	(1.38)	(1.20)	(1.48)	(2.70)	(2.42)	(2.00)	(0.78)	(2.21)	(0.78)

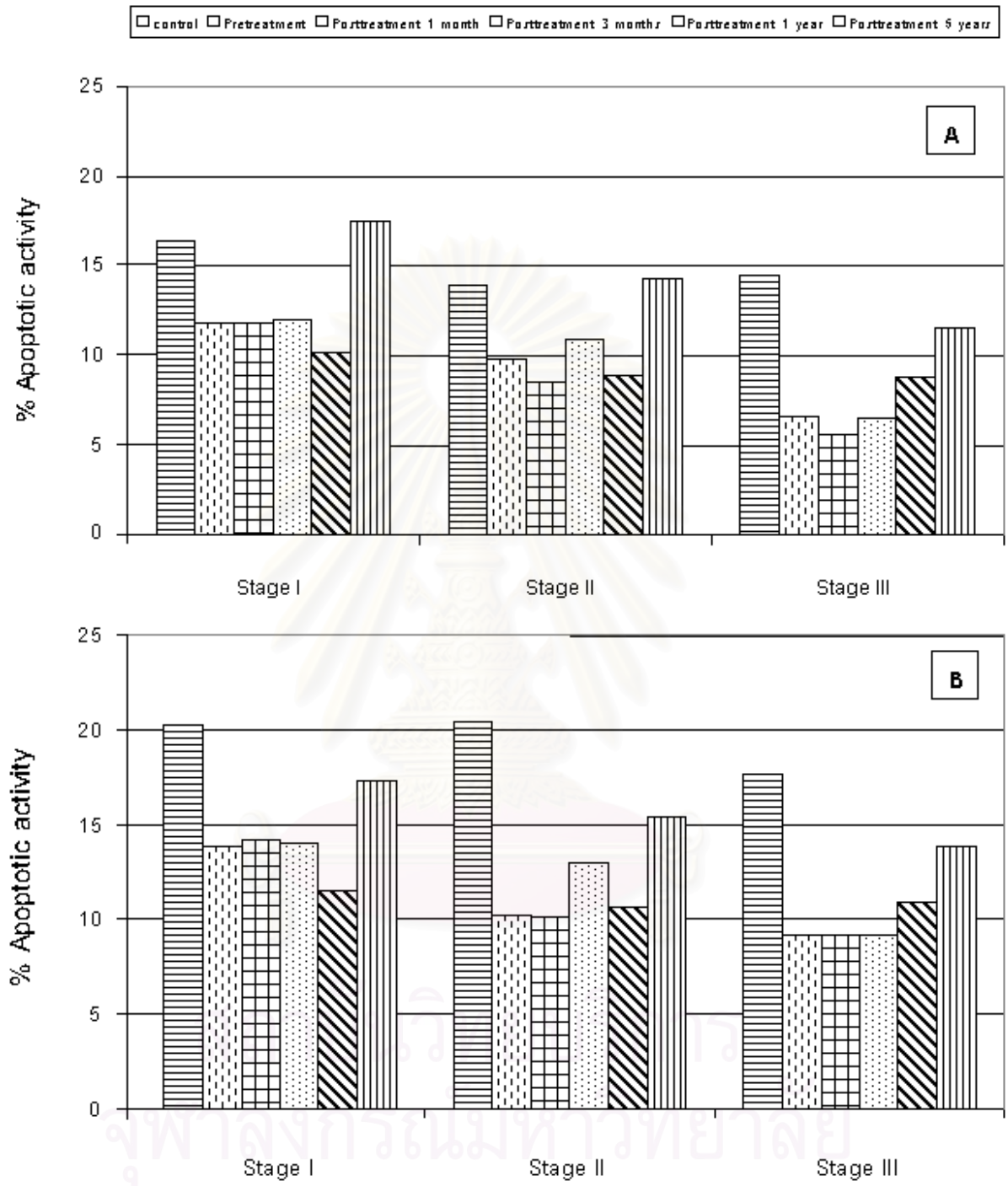


Figure 26 Comparison of the mean of percent apoptotic activity in lymphocytes between cervical cancer Stage I, II and III at various time intervals. The mean of the control group of each stage was included. The apoptotic cells were detected by TdT assay at 24 hours (A) and 48 hours (B) after low dose irradiation.

CHAPTER V
DISCUSSION AND CONCLUSION

Lymphocyte is a nuclear hematocyte which can be induce by low dose of radiation to undergo apoptosis. In this study, lymphocytes were induced by 0.5 Gy of Co radiation that can cause damage to the DNA of lymphocyte after the exposure to the ionizing radiation. The damage results in a fragmentation of DNA within cells that are undergoing apoptosis. [5,6,56,67,70] There are many methods used for detection of cell apoptosis. Some investigators use morphologic criteria, [62,65] while others use methods to detect changes in the integrity of DNA through pulsed-field gel electrophoresis [63-65] or TdT assay.[5,63,65,66] There are strong evidences that DNA fragmentation can be detected by the labeling of apoptotic nuclei using the TdT assay.[5,63,65,66] This assay is a rapid and sensitive method.[5] Another researcher has found that TdT assay is the most accurate tool for detecting DNA damage, although it is still difficult to carry out a quantitative analysis in an *in situ* study. [74] Many studies have shown that apoptosis in lymphocytes requires many hours to develop. They suggest that 48 hours post-irradiation induction will give a high percent of apoptotic cells and a significance of apoptotic yield.[5,7,70] These results agree with our study. The evidences can be found in Table 4 for Stage I; Table 5 for Stage II; and Table 6 for Stage III, where the means of percent apoptotic activity at 48 hours post low dose irradiation in all stages and at every time intervals are greater than the means at 24 hours. Besides, the means of the 3 control groups (Table 4-6) at 48 hours are greater than that at 24 hours as well.

Concerning the sensitivity of different cell types to low dose radiation induction which may affect our model of study. There are studies that demonstrate that different cell types ie; lymphocytes, skin cell [67],and fibroblast [75] taken from the same individual possess the same level of radiosensitivity. Boreham et al. [5] have postulated that the apoptotic response to low dose radiation of peripheral lymphocytes may be used as a biological dosimeter in each individual. Apoptosis in many cell types is short-lived, but lymphocytes

in cell culture display an arrested apoptosis after radiation exposure.[58] The cells remain in this state for many days and therefore the apoptotic cells accumulate. However, there has been reported that, approximately 0.3 $\pm$ 0.2% of the lymphocytes exhibit apoptotic change immediately after the isolation process. [5] Other report [76] has demonstrated that 8 % of lymphocytes in the cell culture will undergo spontaneous apoptosis. In order to obtain actual apoptotic changes in our study, we have double duplicates of the lymphocyte cultures of each sample. One of the duplicate is irradiated with low dose irradiation, the second duplicate without irradiation is analyzed and counted for spontaneous apoptotic changes or the background. All the results presented in this study are the actual apoptotic activity, since the background or spontaneous apoptotic changes has been substracted. Besides, the aging process may also decrease apoptotic activity in normal individual. Since there are no normal data of apoptotic activity in Thai women to be used as a reference. Therefore, we try to match the age of the blood donor controls as close to the age of cervical cancer patients of each stage as possible. In this study, the mean of percent apoptotic activity in 15 healthy Thai women was 14.93 % (range 9.8 % - 22.85 %) and 19.44 % (range 14.4 % - 29.80 %) detected at 24 hours and 48 hours after incubation respectively.

The results from this study, a comparison of the percent apoptotic activity in lymphocytes taken from cervical cancer stage I, II, and III at various time intervals has demonstrated that, there is a change in the apoptotic activity. The percent apoptotic activity in the pretreatment group of all 3 stages is lower than the controls with or without statistical significances. This phenomenon may be explained via a proposal that, apoptosis may be considered as a carcinogenesis opposition, since, death cells cannot turn into tumor cells. If by any reason, this defensive mechanism is disruption, then, the body will be proned to carcinogenesis. However, there may be more than one defense mechanism and so the mechanism of carcinogenesis. This hypothesis may not be applicable to all. In case of the later stage of cacinoma, where there is a spread of the tumor cell or metastasis, the apoptotic change can be explained via other rationales. Zaghloul et al.[77] propose that, in

case of metastasis, tumor angiogenesis may contribute to a reduction of apoptosis activity. And the ability of tumor cells to undergo apoptosis can either be enhanced or inhibited by neovascularization. But, results from the present study have shown that, the percent apoptotic activity in the pretreatment groups of cervical cancer Stage II, III are significantly lower than the controls. So, metastasis in cervical cancer possibly inhibits apoptosis. [78] This may contribute to another proposal that, the apoptotic activity may be applied as a parameter to detect recurrent of cervical cancer in long term follow up. Moreover, our results show the spontaneous apoptosis in lymphocytes that the spontaneous apoptosis in pretreated groups were lower than the control groups without statistical significances in all stages. After radiation therapy, the spontaneous apoptosis in the patients was increased. Wheeler et al. [68] found that cervical cancer patients with high level of apoptosis had better survival. So, high spontaneous apoptosis may be a useful predictor of response to radiation therapy in cervical cancer.

The percent apoptotic activity in the posttreatment groups all 3 stage shows higher increases with longer intervals after the completion of radiation therapy. Never the less, at 1 year interval, the percent apoptotic activity in lymphocytes of all 3 stage of cervical cancer decreases again. But most of the patients do not exhibit any recurrent symptoms. The common complaints include, constipation and slight edema of the lower limbs. Literature reviews do not yield any satisfactory answers to this phenomenon. It might be possible that, after exposure to high dose radiation during the treatment period, most of the radiation sensitive tissues including the tumor have been cleared away. The more periphery cells and tissues have been sensitized by lower dose radiation are still survived. These cells including the lymphocytes have developed the radiation resistant property. These, the percent apoptotic activity then decrease.

The results of percent apoptotic activity in the 5 year posttreatment groups indicate an increase of the percent apoptotic activity towards the level found in the control groups. Several studies have shown that high levels of spontaneous apoptosis are

associated with improved survival of cervical cancer patients. [68,79,80] However, there is variability between persons for the radiation induced apoptosis in lymphocytes. And individual responses can change over a longer time period or be modified by factors such as health status, environmental stress, and life style.[5] In addition, hyperthermia treatments, rise of the body temperature equivalent to a fever, can also modify the response of lymphocytes to radiation-induced apoptosis.[81] It seem that, apoptosis plays an important role in response to radiotherapy. It may also be a marker for the biological aggressiveness of tumors. Further research efforts are needed for understanding the loss of apoptotic response invariably that occurs during solid tumor evolution in hope of providing more effective cancer therapy. However, apoptosis in lymphocytes needs to be evaluated as a potential predictive assay for measuring tissue radiosensitivity that it could to improved tumor cure rates by enabling radiation doses to be tailored to the individual.

In summary, this study has showed that the percent apoptotic activity in lymphocytes taken from pretreated cervical cancer patients is lower than the control groups. After radiation therapy, the percent apoptotic activity in all 3 stage of cervical cancer has increased towards the control groups.

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APPENDICES

APPENDIX A

Buffers and Reagents

1. RPMI 1640 stock solution 1 liter

   RPMI powder

   Na HCO_3

   ddH_2O

   Adjust pH to 7.2 with 1M HCl.

   Add ddH_2O to 1 liter and sterilized by filtering through a 0.45 μm membrane filter.

2. HBSS stock solution 1 liter

   HBSS powder

   $NaHCO_3$

   ddH_2O

   Adjust pH to 7.2 with 1M HCl.

   Add ddH_2O to 1 liter and sterilized by filtering through a 0.45 μm membrane filter.

3. 10X Phosphate Buffered Saline (PBS) 1 liter

   NaCl

   KCl

   Na_2HPO_4

   KH_2PO_4

   ddH_2O

   Adjust pH to 7.4 with 1M HCl.

   Add ddH_2O to 1 liter and sterilize by autoclaving.

4. 1X Phosphate Buffered Saline (PBS) 1 liter

   10XPBS

   ddH_2O

   Sterilize by autoclaving.

5. 10 % Buffered Formalin Acetate solution 1 liter

   Paraformaldehyde powder

   1 M NaOH

   Formaldehyde

   10 X PBS

   warm ddH_2O

   Adjust pH to 7.00 with 1 M HCl.

   Add ddH_2O to 1 liter and sterilized by filtering through a 0.45 μm membrane filter.

6. PBS / 1 % Paraformaldehyde fixative 1 liter

   1 X PBS

   10 % Buffered Formalin Acetate

   Adjust pH to 7.1 and sterilized by filtering through a 0.45 μm membrane filter.

7. 200 mM L-Glutamine

   L-Glutamine

   ddH_2O

   Steriliz by filtering through a 0.45 μm membrane filter.

   Store 5.2 ml in polyporpylene centrifugue tube at -20 ^{0}C.

   ** Do not heat; Since heat will destroy glutamine.

8. Hoeschts 33258 Dye stocksolution

   Hoeschts dye

   ddH_2O

   Store in polypropylene centrifuge tube at -20^{o}C.

9. Hoeschts Dye solution (for 8 slide)

Hoeschts Dye 33258 stock solution

1 X PBS

** Prepare freshly before use.

10. 2 μl / ml Heparin in HBSS

HBSS stock

Heparin

11. Complete RPMI 1640 medium

RPMI stock

L-Glutamine

Gentamycin

Fetal Bovine Serum

12. HBSS / 2 μl /ml Heparin / 1 % Fetal Bovine Serum

HBSS

Heparin (5,000 i.u./u.i./ ml)

Fetal Bovine Serum

13. Stop Wash Buffer (Apoptag kit)

Stop Wash Buffer

ddH_2O

Stored at 4^{o}C.

14. TdT mixture (for 8 Slides)

Reaction Buffer

TdT enzyme

** Prepare freshly before use.

15. FITC mixture (for 8 slides)

    Blocking solution

    Anti-Digoxygenin

    ** Prepare freshly before use.

16. Hoeschts Dye solution

    Hoeschts Dye solution

    1 X PBS

    ** Prepare freshly before each use.

17. 1 M NaOH

    NaOH

    ddH_2O

    Store at room temperature.

APPENDIX B

Results of statistical analysis

The statistics used for the analysis of the percent apoptotic activity in this study were unpaired t test and one way ANOVA. The percent apoptotic activity in lymphocytes detected at 24 and 48 hours after induction by low dose radiation were separately analyzed.

A: Stage I, 24 hours

Control group versus pretreated group	p = 0.116
Control group versus posttreated 5 year group	p = 0.713
pretreated group versus posttreated 1 month group	p = 0.994
pretreated group versus posttreated 3 month group	p = 0.936
pretreated group versus posttreated 1 year group	p = 0.523
pretreated group versus posttreated 5 year group	p = 0.032 *

Stage I, 48 hours

Control group versus pretreated group	p = 0.168
Control group versus posttreated 5 year group	p = 0.507
pretreated group versus posttreated 1 month group	p = 0.894
pretreated group versus posttreated 3 month group	p = 0.953
pretreated group versus posttreated 1 year group	p = 0.375
pretreated group versus posttreated 5 year group	p = 0.209

* The mean difference is significant at the .05 level.

B: Stage II, 24 hours

Control group versus pretreated group	p = 0.075
Control group versus posttreated 5 year group	p = 0.883
pretreated group versus posttreated 1 month group	p = 0.589
pretreated group versus posttreated 3 month group	p = 0.630
pretreated group versus posttreated 1 year group	p = 0.688
pretreated group versus posttreated 5 year group	p = 0.063

Stage II, 48 hours

Control group versus pretreated group	p = 0.001 *
Control group versus posttreated 5 year group	p = 0.079
pretreated group versus posttreated 1 month group	p = 0.964
pretreated group versus posttreated 3 month group	p = 0.128
pretreated group versus posttreated 1 year group	p = 0.782
pretreated group versus posttreated 5 year group	p = 0.007 *

* The mean difference is significant at the .05 level.

C: Stage III, 24 hours

Control group versus pretreated group	p = 0.024 *
Control group versus posttreated 5 year group	p = 0.252
pretreated group versus posttreated 1 month group	p = 0.248
pretreated group versus posttreated 3 month group	p = 0.686
pretreated group versus posttreated 1 year group	p = 0.162
pretreated group versus posttreated 5 year group	p = 0.001 *

Stage III, 48 hours

Control group versus pretreated group	p = 0.000 *
Control group versus posttreated 5 year group	p = 0.015 *
pretreated group versus posttreated 1 month group	p = 0.967
pretreated group versus posttreated 3 month group	p = 0.958
pretreated group versus posttreated 1 year group	p = 0.089
pretreated group versus posttreated 5 year group	p = 0.000 *

* The mean difference is significant at the .05 level.

D: Stage I : Stage II : Stage III

Time interval of treatment	Comparison	P value	
		24 hours	48 hours
pretreatment	Stage I versus Stage II	0.284	0.083
	Stage I versus Stage III	0.014 *	0.034 *
	Stage II versus Stage III	0.104	0.628
Posttreatment 1 month	Stage I versus Stage II	0.050	0.057
	Stage I versus Stage III	0.001 *	0.024*
	Stage II versus Stage III	0.760	0.644
Posttreatment 3 months	Stage I versus Stage II	0.682	0.635
	Stage I versus Stage III	0.060	0.047 *
	Stage II versus Stage III	0.123	0.109
Posttreatment 1 year	Stage I versus Stage II	0.355	0.428
	Stage I versus Stage III	0.339	0.572
	Stage II versus Stage III	0.973	0.815
Posttreatment 5 years	Stage I versus Stage II	0.244	0.417
	Stage I versus Stage III	0.041 *	0.150
	Stage II versus Stage III	0.310	0.499

* The mean difference is significant at the .05 level.

APPENDIX C

The spontaneous apoptosis in lymphocytes detected at 24 hours and 48 hours after induction by low dose radiation were demonstrated.

A: Stage I

Subjects	Control group		Pretreatment		Posttreatment							
					1 month		3 months		1 year		5 years	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	11.95	15.70	10.50	11.40	7.20	8.15	11.0	12.95	9.80	11.00	10.00	13.55
2	10.85	14.95	6.25	7.10	3.80	6.90	6.55	9.15	13.90	14.65	7.20	9.25
3	8.50	11.85	8.90	9.10	12.40	13.80	12.95	14.60	9.45	10.15	11.60	15.75
4	9.30	13.25	7.90	8.50	11.95	12.00	4.35	8.70	6.90	7.40	8.25	10.65
5	8.55	11.95	6.70	7.80	6.10	9.70	2.75	5.25	6.95	7.65	9.50	11.00
mean	9.83	12.11	8.05	8.70	8.29	10.11	7.52	10.13	9.40	10.17	9.31	12.04
SD	1.52	0.86	1.72	1.65	3.76	2.81	4.34	3.70	2.86	2.95	1.68	2.59

B: Stage II

Subjects	Control group		Pretreatment		Posttreatment							
					1 month		3 months		1 year		5 years	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	14.15	16.15	7.10	9.15	11.25	13.00	14.00	15.05	9.75	11.50	10.25	12.00
2	10.85	11.20	9.20	10.40	9.35	10.15	9.70	12.45	10.60	12.55	9.95	11.95
3	9.30	11.70	11.45	12.35	4.55	8.80	8.25	9.95	8.95	9.95	11.15	14.45
4	13.10	13.65	6.60	7.55	6.75	7.10	7.90	8.15	9.25	11.00	8.70	12.55
5	12.35	13.05	4.60	6.95	7.70	9.75	8.95	11.00	10.70	12.85	7.15	8.50
mean	11.95	13.15	7.79	9.28	7.92	9.76	9.76	11.32	9.85	11.57	9.44	11.75
SD	1.91	1.95	2.62	2.19	2.54	2.16	2.47	2.61	0.79	1.18	1.55	2.17

C: Stage III

Subjects	Control group		Pretreatment		Posttreatment							
					1 month		3 months		1 year		5 years	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	8.95	9.95	8.30	9.70	13.00	14.45	11.35	12.25	7.60	8.95	9.35	10.35
2	10.15	11.35	5.75	6.65	10.40	12.10	12.70	12.95	6.55	8.00	11.25	12.20
3	8.85	10.00	10.20	10.95	6.90	7.95	10.90	11.15	7.25	8.25	13.80	14.15
4	7.90	9.25	7.50	8.15	6.80	8.25	4.55	6.65	9.95	11.10	8.95	9.25
5	5.15	7.15	7.55	8.30	6.95	8.85	6.25	8.25	11.25	12.50	10.25	12.10
mean	8.20	9.54	7.86	8.75	8.81	10.32	9.15	10.25	8.52	9.67	10.72	11.61
SD	1.88	1.54	1.61	1.64	2.79	2.84	3.54	2.70	1.99	2.01	1.94	1.88

Biography

Miss Pisut Pamutha was born on May 30,1975 in Ubonrachathanee province, Thailand. She received the degree of Bachelor of Science in Biology, 1997 from Faculty of Science, Srinakarinvirot Prasanmit University, Bangkok, Thailand. She has enrolled at Chulalongkorn University in graduate programme for the degree of Master of Science in Medical Science and graduated in 2000.