I. INTRODUCTION

1.1 Statement and significance of problem

Thalassemia is a group of genetic disease where hemoglobin synthesis is impaired. This chronic anemia leads to increase dietary iron absorption, which develops into iron overload pathology. Treatment through regular transfusions increases oxygen capacity but also provides iron through the hemoglobin of red cells. An essential treatment, in parallel with transfusions, is the use of chelating agents to remove the excess iron deposited in tissues. These deposits are found in the liver, spleen, heart, and pancreas and are associated with cardiac failure and diabetes. Thalassemic patients are particularly at risk of free radical induced damage.

Free radicals capable of direct oxidative damage to macromolecules including DNA, protein, and lipid membrane. An effect of excess results of oxygen free radicals, such as DNA strand breaks and membrane blebbing, match the hallmark feature. Apoptosis is a mode of cell death involved in many physiological processes and several pathological conditions. The cells could be induced to undergo apoptosis or necrosis depending to the extent or intensity of stimulants. However, it was a dose dependent manner in apoptotic pathway.

It was reported that endothelial cells when incubated with α- and β-thalassemic serum, the number of proliferating cells was decreased. It seems to be correlated with the clinical signs and symptoms of thalassemic patients who are developed leg ulcer and pulmonary thromboembolism. The main suspicious target(s)

in the serum was complement, free radical or the oxidized LDL, the high level of free plasma iron or modified LDL (Banjerdpongchai R, *et al.*, 1997).

Recently, an effect of iron over load on cell oxidatively stressed with hydrogen peroxide (H_2O_2) has been determined. Iron over load was stimulated with ferric chloride ($FeCL_3$) and ferrous sulphate ($FeSO_4$). Human lymphocytes of male and female donors and human adenocarcinoma colonic cells were showed an increase in DNA damage in the Comet assay after treatment with H_2O_2. Ferric chloride produced an increase in DNA damage in human colonic cells, but little or no damage in human lymphocytes, but ferrous sulphate produced a dose-related response. When H_2O_2 was combined with $FeCL_3$ or $FeSO_4$, the DNA damage produced was slightly greater than with H_2O_2 alone (Anderson D, *et al.*, 2000).

In this study was investigated, as a model system, apoptosis of nucleated red blood cell (NRBC) and peripheral blood mononuclear cell (PBMC) of β-thalassemic patients were measured by comet assay and cells were treated with ferrous sulphate ($FeSO_4$) and hydrogen peroxide (H_2O_2) for measured the effect of iron on cell oxidatively with hydrogen peroxide. Activity of antioxidants (curcumin) to protect cell oxidatively stressed and apoptosis were also studied.

1.2 Literature reviews

1.2.1 Thalassemia prevalence and clinical manifestation

Thalassemia is a hereditary anemia disease, which is characterized by insufficient synthesis of either, α or β-globin synthesis (Weatherall DJ and Clegg JB, 1981). It is the most common genetic disorder among the people living in Southeast Asia. α-Thalassemia, β-thalassemia, hemoglobin (Hb) E and Hb constant spring (CS) are prevalent. The gene frequencies of α-thalassemia reach 20-30% in northern part of Thailand (Fucharoen S and Winichagoon P, 1987). β-Thalassemia gene frequencies were varied between 1 and 9% (Fucharoen S and Winichagoon P, 1987). Hb E is the hallmark of Southeast Asia, attaining a gene frequency of 50-60% at the junction of Thailand, Laos, and Cambodia. Hb Constant Spring frequencies were varied between 1-8% (Fucharoen S and Winichagoon P, 1987; Tanphaichitr VS, *et al.*, 1987; Thonglairoam V, *et al.*, 1991). These abnormal genes in different combinations lead to over 60 thalassemia syndromes. The two major α-thalassemia disease are Hb Barts' hydrops fetalis or homozygous α- thalassemia 1 and Hb H disease which could occur from the interaction between α-thalassemia 1 and α-Thalassemia 2 or between α-thalassemia 1 and Hb CS. Interaction between β-thalassemia and β-Thalassemia/Hb E which are major β-thalassemia syndromes in the region (Fucharoen PW and Fucharoen F, 1992).

There is no α-globin chain production in Barts' hydrops fetalis resulting in the most serious form of thalassemia diseases. The fetus dies in utero or soon after birth because Hb Barts' does not release O_2 to the tissues. The affected features are hydropic with severe growth retardation, and abnormal development of vital organs

such as brain and lung contributes to the severe morbidity that makes the condition incompatible with life (Fucharoen S and Winichagoon P, 1987).

Homozygous β-thalassemia causes a severe disease known as Cooley's anemia or thalassemia major. The clinical manifestation of this disease develops in the first year of life. The patients have growth retardation, thalassemic faces and hepatosplenomegaly. Regular blood transfusions are needed to reduce the degree of anemia. β-Thalassemia/Hb E contains a wide rang of clinical severity; the hemoglobin levels range between 3 to 13 g/dl. The patients with very low hemoglobin levels may be as severe as homozygous β-thalassemia. Extramedullary hemopoiesis leads to hepatosplenomegaly, iron overload, infections, leg ulcer, pulmonary thromboembolism, and hypoxemia are among the common complications seen in the thalassemic children (Pootrakul P, *et al.*, 1981).

1.2.2 **Molecular defects of α-thalassemia**

The α-globin gene cluster is located on the short arm of chromosome 16 and contains seven gene arranged in the order ζ-ψ-ψα2-ψα1-α2-α1-θ1 (Weatherall DJ and Clegg JB, 1981). The two α-globin genes α1 and α2 have high degree of structure similarity and produce identical products of α-globin chain. DNA analysis revealed that α-thalassemia is most common due to deletion involving one or both of the duplicated α-globin genes. Deletions that cause α-thalassemia 1 in the Thai usually remove about 20 kb of DNA including both α1 and α2 genes from the α-globin gene cluster (Fucharoen PW and Fucharoen F, 1992). The 5'start of the deletion may exist with the third exon of the ζψ-gene and the 3' end of the deletion terminates close to the hypervariable region located at the 3' end of the α-gene

complex (Winichagoon P, *et al.*, 1984). Deletion of the entire α-globin gene complex ($--^{THAI}$) is rare. In α-thalassemia 2 has only one gene (-α) that is left functioning. Two types of thalassemia 2 have detected. One involves a deletion of the α 2-globin genes with 4.2 Kb of DNA and only the α 1-globin genes is left functioning ($-\alpha^{4.2}$); the other removes 3.7 kb of DNA between the linked α1 and α 2-globin genes and produces hybrid α-globin genes ($-\alpha^{3.7}$). The latter has more distribution and is the most common form of α-thalassemia 2 in Thailand (Fucharoen PW and Fucharoen F, 1992). Study of 406 cord blood samples by Southern blot technique revealed that the incidence of α-thalassemia 1 in Bangkok is 3.5% and for α-thalassemia 2 is 16.25%. Only 5% of these α-thalassemia have the 4.2 kb deletion type (Fucharoen PW and Fucharoen F, 1992).

Since the two α-globin genes (α1 and α2) are embedded within the highly homologous regions, unequal homologous recombination within these regions can lead to α-gene rearrangements and results in a single α-globin (α-thalassemia 2) or a triplicate α-globin genes (Fucharoen PW and Fucharoen F, 1992).

There are four α-globin genes in normal individuals; and four varieties of α-thalassemia can be defined depending on the number of α-globin gene deletions in the diploid genome. Deletion of 1, 2, 3, 4 α-globin genes results in α-thalassemia 2 (-α/αα), α-thalassemia 1(--/-α) and Hb Barts' disease (--/--), respectively. Deletions of 1 or 2 α-globin genes do not give rise to clinical disease (Fucharoen PW and Fucharoen F, 1992).

Hemoglobin CS is a variant with elongated α-globin chain, which arises from the mutation at the termination codon of the α2-globin genes (TAA→CAA). This

results in the longer message, which is translated to the next in phase termination signal and produces a globin with more 31 amino acid instead of 141 amino acids as usual. Although α^{CS}- mRNA can be detected in bone marrow of the patients, it is absent from their peripheral blood suggesting its instability (Hunt DM, *et al.*, 1982). Because of the remarkable reduction in α^{CS}-chain production, Hb CS behaves similar to the α-thalassemia 2 genes and leads to Hb H disease when interacting with α-thalassemia 1 (--/αα). However, the loss in expression of the α2-globin locus by nondeletion mutation appears to be more severe than loss of the α-globin gene in the 37 kb deletion type of α-thalassemia 2 ($-\alpha^{3.7}$). Hb H levels and red blood cells containing inclusion bodies are significantly higher in Hb H-CS (α-thalassemia 1/Hb CS, $--/\alpha^{CS}\alpha$) than in the deletion Hb H (α-thalassemia 1/α-thalassemia 2, --/-α) (Winichagoon P, *et al.*, 1982). Quantitation of α-mRNA also demonstrated a more significant loss of α-globin mRNA synthesis from the $\alpha^{CS}\alpha$ chromosome than from the $-\alpha^{3.7}$ chromosome (Winichagoon P, *et al.*, 1988). Furthermore, homozygosity for Hb CS (α^{CS}/α^{CS}) is symptomatic with mild anemia, jaundice and splenomegaly whereas, homozygous α-thalassemia 2 with the $-\alpha^{3.7}$ deletion type (-α3.7/- α3.7) is asymptomatic (Fucharoen PW and Fucharoen F, 1992).

Hb CS is the most common nondeletion α-thalassemic mutation and is an important cause of Hb H-like disease in Southeast Asia. Analysis of membrane from red blood cells of individuals with Hb H/CS and Hb CS/Cs by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the presence of protein band with the molecular weight was slightly greater than globin. This band was reacted with rabbit anti-human α-globin antibodies in a Western blot assay. The identity of this

membrane-bound α-globin like protein as being α^{CS}-globin was verified by comparing its ratio of biosynthesis [^{3}H]-labeled proline/[^{14}C]-labeled lysine with those of normal globin chains. The presence of α^{CS}-globin on the membrane of Hb H/CS and Hb CS/CS red cells affords an explanation for the unusually severe anemia observed in these patients (Peerapitayamongkol C, *et al.*, 1996).

Hb CS variants have an almost normal mean cell volume (MCV) and the anemia is more severe when compares with other α-thalassemic variants. The underlying cause (s) of this MCV "normalizing" affects of Hb CS and the severe anemia is not fully explained. Hb CS containing RBCs are distinctly overhydrated relative to deletional α-thalassemic variants, and the derangement of volume regulation and cell hydration early occur in erythroid maturation and are fully expressed at the reticulocyte stage. The membrane rigidity and membrane mechanical stability of Hb CS containing RBCs are increased when compares with Hb H and α-thalassemia-1 trait RBCs. The cause(s) underlying these cellular alterations was sought by analyzing membrane from Hb CS and deletion α-thalassemic variants and it was found that in addition to oxidized β-globin chains, oxidized α^{CS}-globin chain are also associated with in the membrane and their skeletons in Hb CS containing RBCs. It has been proposed that the membrane pathology of Hb CS is caused by combination of the deleterious effects induced by membrane-bound of the deleterious effects induced by membrane-bound oxidized α-CS and β-globin chains (Schrier SL, *et al.*, 1997).

1.2.3 **Molecular defect of β-thalassemia**

The β-globin gene cluster is located on the short arm of the chromosome 11 in a 50 kb region contains ε, Gγ, Aγ, φβ, δ and β-globin gene. β-Thalassemia is a very heterogeneous disorder because of various defects in the β-globin genes. Single base substitutions or small deletion or insertions in the nucleotide sequences are mainly responsible for the molecular defects of β-thalassemia. These mutations affect transcription factor interaction, the process of transcription, RNA processing, and RNA translation. Mutations that affect the promoter region of the genes or processing of mRNA and reduce the level of functional mRNA that cause mild β-thalassemia (β^+-thalassemia) while the mutations affecting the abolition of mRNA production or producing the nonfunctional mRNA cause severe β-thalassemia (β^0-thalassemia) (Winichagoon P, *et al.*, 1992).

Seventeen mutations have detected in Thai subjects by using allele specific oligonucleotide (ASO) probes or DNA sequencing techniques (Thein SL, *et al.*, 1988; Pootrakul P, *et al.*, 1988; Petmitr S, *et al.*, 1989; Winichagoon P, *et al.*, 1989 and Winichagoon P, *et al.*, 1992). Among these, four are common, which were firstly, a 4-bp deletion leading to frameshift at codon 41/42 (-CTTT); secondly, a nonsense mutation at codon 17 (A→T); thirdly, a C→T mutation at position 654 at the IVS-2; fourthly, A→G transition at position -28 of the ATA box (Winichagoon P, *et al.*, 1992).

Three mutations had been found in Thai patients: C→A ochre mutation in codon 35, a C→G mutation at position -86 in the promoter region which causes a mild β^+-thalassemia and an insertion of adenine (A) in codon 95 which results in a

shift of the reading frame with terminator at the new codon 101 (Winichagoon P, *et al.*, 1989 and 1992; Thein SL, *et al.*, 1990). The molecular basis of β-thalassemia is still unknown in 3.7% of the patients with β-thalassemia/Hb E and in 10.5% of patients with homozygous β-thalassemia (Fucharoen PW and Fucharoen F, 1992, and Winichagoon WP, *et al.*, 1993).

Hemoglobin E, a β-globin variant ($\beta 26^{Glu \rightarrow Lys}$) commonly found in Thailand behaves like a mild β^+thalassemia. The codon change, GAG→AAG, at position 26 activates an alternative splice site at codon 25 (Orkin SH, *et al.*, 1982). Thus, in addition to normally spliced mRNA the utilization of this cryptic splicing site results in a deletion of a portion of first exon. This mechanism results in reduced β^E-mRNA and hence reduces synthesis of β^E-globin chains accounting for the β^+-thalassemia phenotype of Hb E (Traeger J, *et al.*, 1982).

1.2.4 **Pathophysiology**

It is possible to relate almost all the pathophysiologic features of the thalassemias to a primary imbalance of globin-chain synthesis. It is this phenomenon that makes them fundamentally different from all the other genetics and acquired disorders of hemoglobin production and that extent explains their extreme severity in the homozygous or compound heterozygous states.

In homozygous β- thalassemia, β-globin synthesis is either absent or markedly reduced. The production of an excess of α-globin chain is occurred that α-globin chains are incapable of forming a viable hemoglobin tetramer, which precipitated in red cell precursors (Fessas P, 1963; Bargellesi A, *et al.*, 1968 and Wickramasinghe SN and Hughes M, 1978). The resulting inclusion bodies can be demonstrated by both

light (Bargellesi A, *et al.*, 1968) and electron microscopes (Fessas P, 1963; Bargellesi A, *et al.*, 1968 and Wickramasinghe SN and Hughes M, 1978). In the marrow, precipitation can be seen in the earliest hemoglobinized precursors and through the erythroid maturation pathway (Yataganas X and Fessas P, 1969). These large inclusions are responsible for the intramedullary destruction of red cell precursors and hence for the ineffective erythropoiesis that characterizes all the β-thalassemias. It has been showed that a large proportion of the developing erythroblasts are destroyed with in the marrow in severe cases (Finch CA, *et al.*, 1970). Some red cells are released that are prematurely destroyed by mechanisms that are considered below. β-Thalassemia heterozygotes also have imbalanced globin-chain synthesis. Furthermore, there is a mild degree of ineffective erythropoiesis.

It appears that there are 2 major routes to damage of the red blood cell membrane. Firsts, globin-chain precipitation process, which is the generation of hemichromes from excess α-chains with subsequent structural damage to the red cell membrane, and similar damage mediated through the degradation products of excess α-chains (Rachmilewiz EA, *et al.*, 1980; Schrier SL, 1994 and Weatherall DJ, 1998). Membrane-bound hemichromes create a copolymer, which promote clustering of band 3 in the membrane, first observed in sickle cell erythrocytes and later in the red cells of β-thalassemias. It seems likely that these clusters are opsonized with autologous immunoglobulin G and complement, after which the cells are removed by macrophages. The products of degradation of free α-chains are globin, heme, hemin (oxidized heme), and free iron and which also play a role in damaging red cell membranes. Excess globin chains bind to different membrane proteins and alter their structure and function. Excess iron, by generating oxygen free radicals, damages

several red cell membrane components, including lipids and protein, as well as intracellular organelles. Heme and its products can catalyze the formation of a variety of reactive oxygen species, which can produce a damage to the red cell membrane. In β-thalassemia, this leads to a relatively rigid underhydrated red cell. Damage to the red cells may also be mediated during their passage through the spleen due to the presence of rigid inclusion bodies.

While most of β-thalassemia heterozygotes are asymptomatic and have a mild hypochromic anemia, there are more severe forms that are dominantly inherited. Many of these involve mutations are in exon 3 of the β-globin gene. A comparison of the length of abnormal gene products due to nonsense or frameshift mutation in the β-globin genes have suggested a mechanism that explains why most heterozygous forms of β-thalassemia are mild, while those due to exon 3 mutations are more severe (Thein SL, et al., 1990 and 1992). Nonsense or frameshift mutations produce truncated β-chain up to about 72 residues in length are usually associated with a mild phenotype in heterozygotes. It appears that mRNA containing stop or frameshift mutations in its 5' region may not be transported to the cytoplasm. However, many exon mutations produce normal amount of mRNA and long truncated products. It has been suggested that the severe phenotypes associated with them reflect their heme-binding properties and stability. Those with only 72 residues or longer should bind heme, since only helix H its missing. Furthermore, such heme-containing products should have a secondary structure and hence be less susceptible to proteolytic degradation. The lack of helix H, which would expose one of the hydrophobic patches of helix G and the hydrophobic patches of helices E and F, would tend to lead to aggregation of the truncated products. It was suggested, therefore, that the large

inclusions in the red cell progenitors of these patients consist of aggregates of precipitated β-chain products together with excess α–chains, a notion that has been shown to be correct (Ho PJ, *et al.*, 1997). This explains the inclusion bodies in the red cell precursors and the marked degree of dyserythropoiesis that is observed in this interesting conditions.

It is clear, therefore, that the anemia of β-thalassemia has three major components. First, there is most important of ineffective erythropoiesis with intramedullary destruction of a variable proportion of the developing red cell precursors. Second, there is a hemolysis due to destruction of mature red cells containing α-chain inclusions. Third, because of the overall reduction in hemoglobin synthesis, the red cell are hypochromic and microcytic.

Because the primary defects in β-thalassemia in β-chain production, the synthesis of hemoglobins F and A_2 should be unaffected. Fetal hemoglobin production *in utero* is normal. However, fetal hemoglobin synthesis persists beyond the neonatal period in nearly all forms of β-thalassemia. In β-thalassemia heterozygotes, there is an elevated level of hemoglobin A_2. A relative decrease in hemoglobin A occurs due to defective β-chain synthesis and an absolute increased in the output of δ-chains both *cis* and *trans* to the mutant β-globin gene (Weatherall DJ and Clegg JB, 1981, Weatherall DJ, et al., 1994).

The consequences of excess non-α-chain production in the α-thalassemias are quite different. Because α-chains are shares by both fetal and adult hemoglobin. In the fetal, it leads to excess γ-chain production while in the adult is to an excess β-chains production. Excess γ-chain of γ_4 homotetramers is hemoglobin Bart's (Ager JAM and

Lehmann H, 1985); excess β chains of β_4 homotetramers is hemoglobin H (Rigas DA, et al., 1955 and Ho PJ, *et al.*, 1997). The fact that γ_4 and β_4 tetramers are soluble, they do not precipitate to any significant degree in the marrow, and therefore the α-thalassemia are not characterized by severe ineffective erythropoiesis. However, β_4 tetrameters were precipitated as red cell age, with the formation of inclusion bodies (Rigas DA, *et al.*, 1955). Thus, the anemia of the severe forms of α-thalassemia in the adult is due to a shortened survival of red cells. Hemoglobin Bart's is more stable than hemoglobin H and does not form large inclusion.

In the case of β-thalassemia, excess α-chains result in mechanical instability and oxidative damage to a variety of membrane proteins. However, in α-thalassemia, the red cell membranes are hyperstable and there is no evidence of oxidation or dysfunction of this protein. Furthermore, the state of red cell hydration is different in α-thalassemia; accumulation of excess β-chains results increased hydration.

There is another factor that exacerbates the tissue hypoxia of the anemia of the α-thalassemias. Both hemoglobin Bart's and hemoglobin H show no heme-heme interaction and have almost hyperbolic oxygen association curves oxygen at physiologic tissue tensions (Bunn HF and Forget BG, 1986 and Weatherall DJ, 2000).

It follows, therefore, that infants with high levels of hemoglobin Bart's have severe intrauterine hypoxia. This is the major basis for the clinical picture of homozygous α^0- thalassemia, which results in the stillbirth of hydropic infants found in late pregnancy or at term. Oxygen deprivation is reflected by the grossly hydropic state of the infant, presumably due to an increase in capillary permeability, and by severe erythroblastosis.

Deficient fetal oxygenation is probably responsible for the enormously hypertrophied placentas, and possibly for the associated developmental abnormalities, that occurs with the severe forms of intrauterine α-thalassemia with the severe form of intrauterine α-thalassemia (Wasi P, *et al.*, 1974).

1.2.5 **Clinical features**

1.2.5.1 **β and δβ Thalassemias**

The most clinical severe forms of β thalassemia is called thalassemia major. A milder clinical picture is characterized by a later onset and either no transfusion requirements or at least fewer transfusions than are required to treat in the major form of the illnesses, that is designated β-thalassemia intermedia. β-Thalassemia minor is the term used to describe the heterozygous carrier state for β-thalassemia.

1.2.5.2 **β-Thalassemia major**

The homozygous or compound heterozygous state for β-thalassemia, thalassemia major is first described by Cooley in 1925 (Cooley TB and Lee P, 1925).

The clinical course is characterized of severe anemia with frequent complications. These children are particularly prone to infection, which is a common cause of death. Because of increased folate utilization by the hypertrophied marrow, folic acid deficiency frequently occurs. Spontaneous fractures commonly occur as a result of the expansion of the marrow cavities with thinning of the long bones and skull. Maxillary deformities often lead to dental problems from malocclusion. The formation of massive deposits of extramedullary hematopoietic tissue may cause neurologic complications. With the gross splenomegaly that may occurs, secondary thrombocytopenia and leukopenia frequently develop leading to a feature tendency in

the absence of thrombocytopenia. Epistaxis is particularly common. These hemostatic problems are associated with poor liver function in some cases. Chronic leg ulceration may occur, although this is more common in thalassemia intermedia.

Children who have grown and developed normally throughout the first 10 years of life as a result of regular blood transfusion begin to develop the symptoms of iron loading as they enter puberty particularly if they have not received adequate iron chelation. The first indication of iron loading is usually the absence of the pubertal growth spurt and a failure of the menarche. Over the succeeding years, a variety of endocrine disturbances may develop, particularly diabetes mellitus, hypogonadotrophic hypogonadism, and growth hormone deficiency; hypothyroidism and adrenal insufficiency also occur but are less common. Toward the end of the second decade, cardiac complications arise, and death usually occurs in the second or third decade as a result of cardiac siderosis. This may cause an acute cardiac death with arrhythmia, or intractable cardiac failure. Both of these complications may be precipitated by intercurrent infection.

Even the adequately transfused child who has received chelation therapy may suffer a number of complications. Blood-borne infections, notably with hepatitis C (Wonke B, *et al.*, 1998) or HIV (Girot R, *et al.*, 1991, Chanarat P, *et al.*, 1990), are extremely common in some populations, although their frequency is being reduced by the use of widespread blood-donor screening programs. Delayed puberty and growth retardation is also common and probably reflect hypogonadotrophic hypogonadism together with damage to the pituitary gland (Wonke B, *et al.*, 1998; Chatterjee R, *et al.*, 1993). Osteoporosis is also being increased and may also be a reflection of hypogonadism (Wonke B, *et al.*, 1998).

1.2.5.3 **β-Thalassemia intermedia**

The clinical phenotype of patients designated to have thalassemia intermedia is more severe than usual symptomatic thalassemia trait but milder than transfusion-dependent thalassemia major (Weatherall DJ, *et al.*, 1985, Wainscoat JS, *et al.*, 1987 and Weatherall DJ, 1993). The syndrome encompasses disorders with a wide spectrum of disability. At the severe end, patients present with anemia later than usual in transfusion-dependent forms of homozygous β-thalassemia and are just able to maintain a hemoglobin level of about 6 g/dl without transfusion. However, their growth and marked skeletal deformities, arthritis, bone pain, progressive splenomegaly, growth retardation, and chronic ulcerations above the ankles are observed. At the other end of spectrum, there are patients who remain completely asymptomatic patient whom are transfusion-independent, with hemoglobin level as high as 10 to12 g/dl. All varieties of intermediate severity are observed, and some patients become disabled simply due to the effects of hypersplenism. Intensive studies of the molecular pathology of this condition have provided some guidelines about genotype-phenotype relationship that are useful for genetic counseling.

1.2.5.4 **β-Thalassemia minor**

The heterozygous state for β-thalassemia is not usually associated with any clinical disability, and the abnormality is discovered only on performing a blood examination. It is most commonly discovered during periods of stress, such as pregnancy or during severe infection, when moderate degree of anemia may be found. Some patients with thalassemia minor have increased iron stores, but often this may be due to injudicious iron therapy started because of misdiagnosed microcytic anemia.

1.2.6 **Thalassemia and oxidative stress**

In thalassemia the oxidative damage *via* free radical formation (Saltman P, 1989), lipid peroxidation (Chiu D, *et al.*, 1989) and iron toxicity (Hebbel PR, 1990) has been elucidated. The mechanisms facilitating oxidative damage are multifactorial, and result from the presence of excess unpaired globin chains, high intercellular content of non-hemoglobin iron, and low concentration of normal hemoglobin encountered in thalassemic red blood cells (RBCs).

As direct result of genetic defect, thalassemic RBCs contain an excess amount of hemoglobin subunit which is α-globin chains accumulate in β-thalassemic RBCs and β-globin chains of α-thalassemic RBC (Nathan DG and Gunn RB, 1966). Following oxidation, these unstable hemoglobins are known to generate *in vitro* free oxygen radicals. These free radicals form were more methemoglobin and then reversible and were irreversible hemichromes. Finally, they disintegrate to heme and globin moieties, loading the RBC membrane with denatured globin chains, heme and iron (Saltman P, 1989).

Free heme is capable of interacting with either lipid bilayer or with cytoskeletal membrane proteins such as spectrin, actin, and protein 4.1 (Sears DA and Luthra MG, 1983). Hemin degradation is accompanied with iron release, which plays a significant role in RBC oxidation.

Trace metals, e.g. copper and iron, have been implicated as causative agents in excessive generation of endogenous free oxygen radicals, which are capable of causing oxidative stress. In thalassemic RBCs, non-hemoglobin iron is increased. Free irons or aggregates of ferritin and deposits of hemosiderin are catalysts of lipids and protein peroxidation *via* Fenton reaction (Hebbel RP, 1985) which is:

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$$

Data have been accumulated suggesting that increased lipid peroxidation takes place in thalassemic RBCs. The organization of membrane phospholipids in thalassemic RBCs is depicted with most of phosphatidylcholine (PC) being present in the outer bilayer leaflet, and phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner leaflet (Wolfe LD, *et al.*, 1982). Not only, the distribution of membrane lipids were abnormal, but a lower percentage of PE could be detected together with a decrease in the percentage of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (Polliac A, *et al.*, 1974). The flip-flop of phosphatidylserine to the outer surface has also been found (Rachmilewitz EA, *et al.*, 1985). Both PE and the PUFAs are known to be susceptible to peroxidation (Jacob HS and Lux SE, 1968). Another indicator is the high malonyldialdehyde (MDA) contents of thalassemic RBCs detected following exogenous oxidant stress. MDA is the result of PUFA oxidation. MDA is formed from the breakdown of polyunsaturated fatty acids and serves as a convenient index for the determining the extent of peroxidation reaction. It reacts with thiobarbituric acid to give a red species of absorbance of 535 nm (Stocks J, *et al.*, 1972).

Lipid peroxidation is known to case the polymerization of membrane components, thus decreasing cell deformability. Increased membrane rigidity is detected in RBCs from patients with both α- and β-thalassemia. This could be explained by excess globin chains attached to membrane components and MDA which caused the crosslinking of membrane proteins (Pfafferott C, *et al.*, 1982). Lipid

peroxidation is known to play an important role in RBC removal by reticuloendothelial system because of the abnormality in membrane integrity.

Membrane proteins of thalassemic RBCs are damaged by oxidative stress *via* increased crosslinking of membrane proteins and decreased in titratable membrane thiols (Kahane I, *et al.*, 1978). Electrophoretic analysis of RBCs suggests that the RBC membrane skeleton must be damaged. Cytoskeletal network is considered to be important in regulating different RBCs function. Major cytoskeletal proteins are sensitive to oxidants because of topographical proximity of the sulfhydryl groups to RBC membrane PUFAs. The type of abnormalities in RBC cytoskeletal membrane protein is different between α- and β-thalassemia due to the distinct properties of globin chains. In α-thalassemia, the precipitated β subunits (H bodies) are larger and bind more easily to their natural binding site on the membrane the cytoplasmic domain of protein 3- and may mechanically distort the spectrin binding sites.

In α-thalassemia, the morphological appearance of Heinz bodies or H bodies has been attributed to the unequal synthesis of globin chains. They are unstable and trend to precipitate or convert to hemichromes, upon oxidation (Pasteranack GR and Leto TC, 1985). The pathophysiological role of hemichrome in RBC membrane is to promote clustering of band 3 and other membrane proteins. The hemichromes aggregates contain globin chains, membrane proteins (i.e. band 3, ankyrin, protein 4.1, 4.9, actin and glycophorin A or B)

It was demonstrated that membrane sialoglycoprotiens are the site to protect thalassemic RBCs from galactosyl exposure, which would enhance the removal of RBCs from the circulation *via* the immune reaction. Antiprotein 3 is also responsible for removal of RBCs *via* hemoglobin denaturation, hemichrome formation and the

consequent clustering of protein 3. Phagocytosis is increasingly encountered in pathological RBCs or RBCs that undergoes experimental oxidation. Thalassemic RBCs are phagocytzed more readily than control RBCs by macrophages (Knyszynski A, *et al.*, 1979).

Hemoglobin denaturation to hemichrome following oxidation, increased membrane-bound hemichromes and the integral-membrane protein clustering may generate new antigenic site that enables specific binding to autoantibodies, thus leading to ultimate removal of those RBCs from the circulation by reticuloendothelial system. The effects on α-thalassemia 4.1 protein and α-thalassemia ankyrin still need to be warranted since the oxidants are proved to be present in the thalassemia serum *via* the RBC hemolysis, insufficient phagocytosis, and the inflammatory process.

Reactive oxygen species (ROS) are involved in triggering apoptosis, and Bcl-2 may block either the formation of oxygen radicals or their effects (Barinaga M, 1994). Subcellular fractionation studies and laser scanning confocal microscopy indicated that the majority of Bcl-2 within B cell lines is localized as an integral mitochondrial, endoplasmic reticulum and nuclear membrane protein, which are the site of ROS generation.

One of product of mitochondrial metabolism that is potentially injurious to cellular constituents is the production of $O_2^{\cdot}$. Mitochondrion is believed to be a major site of ROS *in vivo*. The principal loss of electrons that converts O_2 to $O_2^{\cdot}$ are involved coenzyme Q, ubiquinone, and its complexes. Another site of electron transport is the endoplasmic reticulum, in which the reduced form of NADH cytochrome 450 reductase leaks electron to O_2 reducing it to $O_2^{\cdot}$.

Direct exposure of various cell types to oxidants such as hydrogen peroxide or lipid hydroperoxide can directly induce apoptosis while in many experimental models, pretreatment of cells with antioxidants has been shown to protect against programmed cell death. The multiple forms of thymocyte apoptosis can be inhibited by free radical spin traps, spin probes, and thoil reductants, and this inhibition correlates with a lower oxidative burden within the treated cells. Possible phospholipase A_2 activated arachidonic acid metabolism. Electron transport factors and inhibitory proteins must be tagged for proteolysis before apoptosis can commence (Orrenius S, *et al.*, 1996).

Hydroxyl radicals ($OH^{\bullet}$) are the most reactive oxygen free radical species capable of direct oxidative damage to macromolecules including DNA, protein, and lipid membrane. Of note, the effect of excess results of oxygen free radicals, such as DNA strand breaks and membrane blebbing, match the hallmark feature of apoptosis. The production of $OH^{\bullet}$ mediates lipid peroxidation, oxidative damage to DNA and sulfhydryl modification of protein (Halliwell B and Gutteridge JMC, 1984).

Exposure to H_2O_2, which generates hydroxyl radical *via* the Fenton reaction, results in cell death with the apoptotic morphology. When the primary product is O_2^-, which is poorly reactive radical, it does not cause lipid peroxidation. Protonated HO_2^- is somewhat more reactive. Most of them is mediated by reactive hydroxyl radical which is generated by two metal catalyzed reactions, either *via* the Fenton reaction or the Harber-Weiss reaction:

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe2+,\ Cu+} O_2 + OH^{\bullet} + OH^{-}$$

The overexpressed MnSOD (manganese-dependent dismutase) does not protect cells from IL-3 deprivation-induced apoptosis, supporting a role of peroxides and downstream metabolites as the important ROS. More convincingly, overexpressed GSHPx (glutathione peroxidase), an enzyme that is localized in the cytosol and mitochondrial matrix, detoxifies produced from mitochondria, endoplasmic reticulum, and cytosol effectively, and counters programmed cell death (Saltman P, 1989).

One role of ROS may be as signaling molecules that regulates including NF-κ B, Fos/Jun and helix-loop-helix members (Abate C, *et al.*, 1990). The lipid membrane peroxidation is measured for vital damage to cells cause by ROS. Thiobarbituric acid tests can detect free malonyldialdehyde which is the end product following decomposition of lipid peroxides of aldehydes.

Cells with Bcl-2 overexpressed still generate peroxides but do not damage their constituents including lipid membranes. A role for Bcl-2 in antioxidant pathway is to act after the generation of $O_2^{\bullet-}$ and its conversion to peroxides and to prevent cellular damage e.g. lipid peroxidation. It is supported by the model of Bcl-2 deficient mice that develop two potentially redox related pathologies: polycystic kidney disease and hypopigmentation (Veis DJ, *et al.*, 1993). Overexpression of GSHPx, an inhibitor of lipid peroxidation, also represses programmed cell death. One plausible role for Bcl-2 would be as a free radical scavenger serving as a non-reactive free radical trap.

Free radicals, such as $O_2^{\bullet-}$, $OH^{\bullet}$, nitric oxide (NO) and H_2O_2, are formed *in vivo*. Imbalance between production of ROS and antioxidant defense results in oxidative stress. Deficiency of antioxidants e.g. glutathione (GSH), ascorbic acid or α-tocopherol and/or increase formation of ROS causes oxidative stress. The ROS found in the blood circulation are formed by the neutrophils and macrophages in the process of immunity and inflammation. Moreover, the oxidant capacity of lipoproteins is evidenced e.g. oxidized LDL (Balla G, *et al.*, 1991). The leakage of the red cell contents such as Fe^{2+} and hemin has been found to induce the oxidative damage or trigger ROS production.

The role of Fe^{2+} on oxidant injury to the endothelium is intriguing since Fe^{2+} is a catalyst capable of simulating ROS generation, e.g. $OH^{\bullet}$. The iron is stored in cell as low molecular weight iron chelates, heme associated iron, ferritin bound iron in the form of Fe^{3+}, and non-transferin iron.

Oxidative stress is important both in cell cytotoxicity and in cellular activation (Schwartzman RA and Cidlowski JA, 1993). The hydrogen peroxide can activate the transcription factor NF-κB (McConkey DJ, *et al.*, 1994). It plays roles in apoptosis. Superoxide radical, $O_2^{\bullet-}$, in neurons is induced by nerve growth factor (NGF) deprivation. Antioxidants have an inhibitory effect on thymocyte apoptosis induced by chemicals. However, studies on highly hypoxic condition indicated that oxygen radicals are not generally required for apoptosis (Muschel RJ, *et al.*, 1995). NO is also important in inducing apoptosis. NO can amplify calcium-induced gene transcriptional in nerve cells (Genaro AM, *et al.*, 1995).

Thalassemic serum contains free radicals. The catalyst of ROS formation in the serum is most likely due to be hemin and/or nontransferin bound iron. Plasma iron level is significantly and potentially high due to ineffective erythropoiesis both intravascular and extravascular hemolysis (Pootrakul P, *et al.*, 1988). So, through the Fenton reaction, the free radicals are generated and cause endothelial cell damage. Since there are no signs and symptoms of inflammation in thalassemic patients who develop leg ulcer or pulmonary thrombosis, it is probably that the endothelial cell damage is due to apoptosis not necrosis, which evokes the inflammatory reaction in the surrounding tissue. Thus may go through the oxidative stress mediators.

1.2.7 **Thalassemia and apoptosis**

Apoptosis is a term referring to the cytologically observable changes associated with a process of active, i. e. gene-directed, cellular self-destruction observed in all eukaryotes. The process has been termed also programmed cell death (PCD). Control of apoptosis is thought to be intimately linked with the progression of cells through the cell cycle and this process essentially guarantees a steady-state condition in which cell division is counterbalanced by cell death.

Apoptosis allows selective elimination and swift clearance by phagocytosis of cells from a proliferating cell population. Apoptotic processes are observed, for example, embryonal development, morphogenesis, metamorphosis, in endocrine tissue atrophy, the normal turnover of tissues, and tumor regression. Apoptosis is also a mechanism of safe clearance of unwanted cells during resolution of inflammation . Cellular self-destruction plays a decisive role in the elimination of self-recognizing T-lymphocytes in the thymus. The disruption of normal processes leading to apoptosis

results in illegitimate cell survival and can cause developmental abnormalities and facilitate cancer development.

Cell death by apoptosis considerably differs from cell death by necrosis, which may be a consequence, for example, of injuries complement attacks, severe hypoxia, hypothermia, lytic virus infections, or exposure to a number of toxins and which eventually leads to cell lysis.

Apoptosis is initiated when cells are given sufficient time to organize a number of intracellular events participating in their own destruction. Unlike necrosis, apoptosis requires a functional energy-producing system. The earliest indications of apoptotic cell death are morphological alterations of the cells such as chromatin condensation, disappearance of the nucleolus, and alterations of the cell surface, characterized by the occurrence of blebs. These signs are followed by a margination of the chromatin at the inner surface of the nuclear membrane. Eventually the activation of specific intracellular calcium-dependent endonucleases leads to the fragmentation of DNA, generating fragments of the size of nucleosomes .

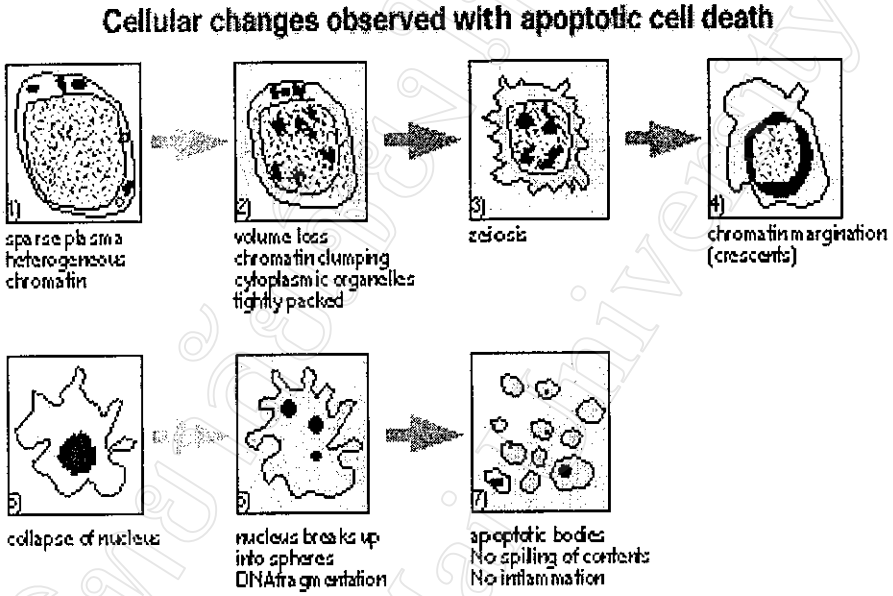

Figure 1. Cellular changes observed with apoptotic cell death.

(www.copewithcytokines.de/cope.)

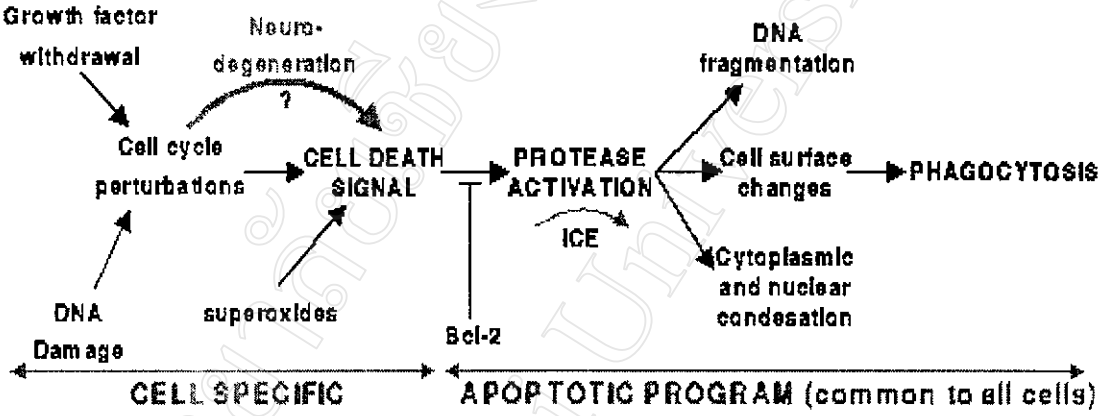

Figure 2. Events in the cell death program. Interleukin-1β-converting enzyme is required for cell death to occur and Bcl-2 protects cells from apoptosis.

In contrast to cell death by necrosis, cells dying by apoptosis shrink and eventually break up into apoptotic bodies. Since intracellular contents are not released from apoptotic cells and their fragments. This process is not accompanied by inflammatory processes and the process, therefore, can be regarded as an injury-limiting mode of cell disposal.

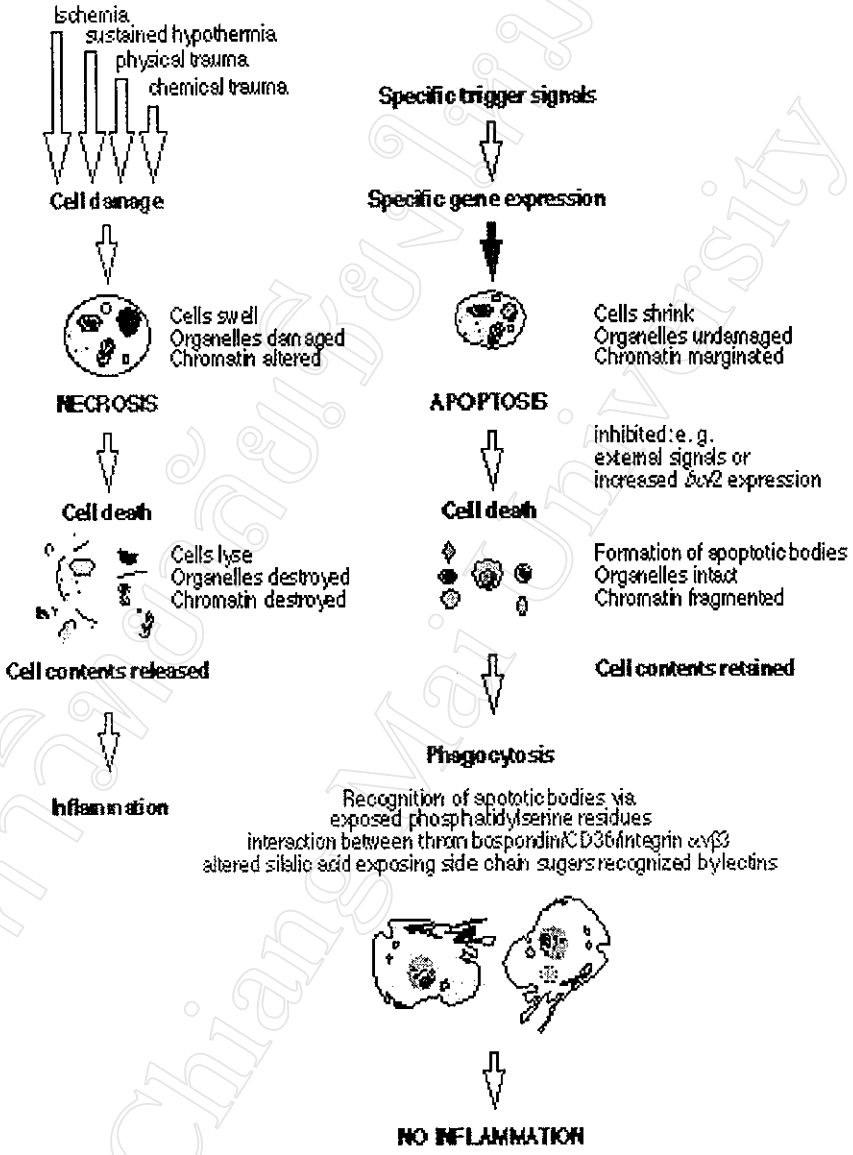

Figure 3. Mechanism of cell death between apoptosis and necrosis.

(www.copewithcytokines.de/cope.)

1.2.7.1 **Comparison of cell death by necrosis and apoptosis**

Cellular necrosis is usually caused by cell damage and does not require further gene activity. The membrane is the major site of damage and, among other things, loses its ability to regulate osmotic pressure. Eventually cell contents are released and elicit inflammatory reactions. Apoptotic cell death requires gene activity. It can be prevented by increased expression of some genes or by external signals. Apoptotic cells eventually break up into apoptotic cell bodies. Cell contents are not released and there is no inflammation.

Being an active process apoptotic cell death can be regarded as a differentiation event. Apoptotic processes involve activators apoptosis inducing factors (AIF), effectors, and negative regulators. The apoptotic process can be inhibited by inhibitors of RNA and protein synthesis, suggesting that a number of specific proteins are required for its initiation and progression. The genetic analysis of some genes of the nematode *Caenorhabditis elegans* has revealed the existence of a variety of genes and proteins involved in the control of apoptosis. The expression of these genes is subject to very complex regulatory circuits. Similar genes have been identified in other species, including humans, and some of the proteins encoded by them are highly conserved.

Cell death can be triggered by a variety of stimuli, including γ irradiation, cytotoxic lymphocytes, glucocorticoids, and various cytolytic cytokines , i.e.TNF-α. In thymocytes apoptosis can be induced by treatment with glucocorticoids or irradiation. In certain hematopoietic cell types the growth of which depends on the continuous presence of growth factors.

Apoptosis can be initiated also by cross-linking of the Apo-1 cell surface antigen. Some cells types such as neutrophils or eosinophils are constitutively programmed to undergo cell death by apoptosis.

The process of programmed cell death is often referred to as activation-induced cell (AIA), involving the engagement of cell surface receptors and subsequent signaling processes initiated from these receptors. At the molecular level apoptosis involves a series of intracellular protein-protein interactions mediated by defined sequence motifs found in the signaling molecules. A variety of proteins has been shown to affect the susceptibility of cells to apoptosis. These proteins frequently interact with membrane proteins and intracellular signaling adapter molecules through a so-called death domain. A plethora of proteins with a death domain has been shown to participate in the generation of signaling complexes referred to frequently as death-inducing signaling complex (DISC). Another domain is the so-called death effector domain , and a third sequence motif is known as caspase activation and recruitment domain (CARD).

In many instances the exact interplay between the proteins forming these complexes still remains to be elucidated. It appears, however, that there are several independent pathways by which apoptosis can be initiated or prevented. In addition, the outcome of the death differentiation program depends critically upon the relative amounts of death-promoting and death-inhibiting proteins and their expression may be altered in a stimulus-dependent manner.

Other death proteins appear to encode membrane proteins and a class of genes known as tumor suppressor genes. A plethora of proteases has been identified that interfere with programmed cell death. They are involved in the proteolytic

inactivation of poly - [ADP-ribose] - polymerase (PARP). The activity of PARP is a critical step in the regulated DNA fragmentation observed during apoptosis. PARP catalyzes the ADP-ribosylation of nuclear proteins at the sites of spontaneous DNA strand breaks and thereby facilitates the repair of this DNA damage.

The simplest way to observe this phenomenon *in vitro* is to use a cell permit DNA-staining fluorescent dye such as *Hoechst* 33342, which allows a striking visualization of the chromatin condensation.

1.2.7.2 **Inducers of apoptosis and apoptotic signaling**

Activators of apoptosis include tumour necrosis factor a (TNFa), Fas ligand (FasL), transforming growth factor b (TGFb), Bax (proapoptotic Bcl-2 family members), and glucocorticoids. In addition, aberrant oncogene expression (e.g. c-*myc*), or normal tumour suppressor gene function (such as *p53*) may trigger apoptosis under specific conditions. In many cases, simultaneous conflicting signals for growth stimulation and suppression trigger apoptosis. Most growth factors exert explicitly anti-apoptotic signaling on there target cells. Cytokines regulate survival through their receptors, which trigger a cascade of intracellular signaling. Among the intracellular (noncytokine) factors which have been shown to potently suppress apoptosis are CD40 ligand, viral genes such as E1B from adenovirus, baculovirus p35, and antiapoptotic genes in the Bcl-2 family. A large number of DNA viruses have been demonstrated to encode factors which function to curtail the cellular apoptotic response (presumably a prerequisite for successful viral infection/propagation). A few apoptotic modulators, including FasL and TNF, induce apoptosis which is largely confined to the development and regulation of the immune system.

1.2.7.3 **Fas and TNFa receptor-mediated apoptotic signaling**

Fas receptor (CD95) belongs to a family of receptors including the tumour necrosis factor (TNF) and nerve growth factor (NGF) receptors that utilize related signaling pathways to regulate cell proliferation, differentiation or death. CD95 (Fas/Apo-1), which plays a critical role in T-cell mediated toxicity. Fas serves as a receptor on the cell surface for a ligand (FasL), and the crosslinking of FasL to Fas receptor triggers apoptosis on the target cells. Fas is abundantly expressed in activated mature lymphocytes, and in lymphocytes transformed with human immunodeficiency virus (HIV) or human T cell leukemia virus (HTLV-I); in addition, certain tumor cells also express Fas. The Fas apoptotic pathway is implicated in eliminating unwanted activated lymphocytes or virus-infected cells and its signaling pathway is summarized in Figure 5.

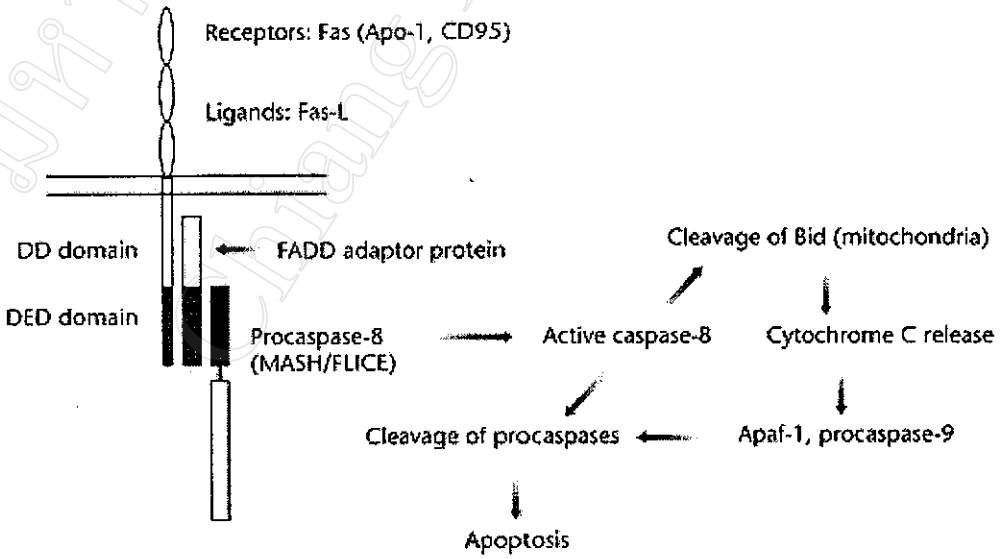

Figure 4. Fas and TNFa receptor-mediated apoptotic signaling.

Fas receptor signaling pathways employ a cytoplasmic protein motif known as the death domain (DD) in the receptors and certain adaptor proteins. The death domain is a conserved sequence of ~80 amino acids. This DD motif is also found in adaptor proteins MORT1/FADD, TRADD and RIP. The death domain of Fas binds to MORT1/FADD. In addition, MORT1/FADD interacts with caspase-8, a member of the ICE/Ced-3 protease family, through another motif designated the death effector domain (DED). Recent evidence suggests that caspase-8 in turn cleaves Bid, a Bcl-2 family protein which may regulate mitochondrial integrity in a manner which further activates the apoptotic cascade

1.2.7.4 **Genes that regulate apoptosis**

The Bcl-2 family of factors regulate caspase activation either negatively (e.g. Bcl-2 itself) or positively (e.g. Bax). Other apoptosis modulators reside further upstream and are thought to activate cascades which are in turn subject to regulation by downstream factors such as Bcl-2. Among these upstream modulators are oncogenes such as c-*myc* which activates apoptosis in a manner which may be important in tumorigenesis or cancer therapy. The tumor suppressor p53 induces apoptosis under certain conditions, thereby accounting for at least a portion of its tumour suppressive activity.

Triggers ⟶	**Regulators** ⟶	**Executioners**
* DNA damage	* p53	* APAF-1
* cytokine starvation	* death domian factors	* caspases
* hypoxia	* Bcl-2 family	
* detachment	* Myc/oncogenes	
* temperature	* cytokine-responsive kinases	
* death receptor	* cytochrome c	

The apoptosis cascade: triggers, regulators and effectors (executioners).

A variety of triggers both pathological and physiological (e.g. during normal development) events can activate apoptosis. Numerous regulators include factors which can dampen or amplify the apoptotic signal, as well as intermediates which are essential participants in a specific apoptotic pathway (e.g. p53). Executioners are activated as downstream effectors. Their activation represents a point of no return in the life or death of a cell.

1.2.7.5 **The Mechanisms of Apoptosis**

There are 2 different mechanisms by which a cell commited suicide by apoptosis. One generated by signals arising within the cell. the other triggered by death activators binding to receptors at the cell surface; TNF, lymphotoxin, Fas ligand (FasL)

1. **Apoptosis triggered by internal signals**

In a healthy cell, the outer membranes of its mitochondria express the protein Bcl-2 on their surface. Bcl-2 is bound to a molecule of the protein Apaf-1. Internal damage in the cell causes Bcl-2 to release Apaf-1 and to no longer keeps cytochrome c from leaking out of the mitochondria. The released cytochrome c and Apaf-1 bind to molecules of caspase 9. The resulting complex of cytochrome c, Apaf-1, caspase 9 and ATP, is called the apoptosome.

Caspase 9 is one of a family of over a dozen caspases. They are all proteases. They get their names because they cleave proteins - mostly each other - at aspartic acid (Asp) residues. Caspase 9 cleaves and activates other caspases. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement activation) which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA and phagocytosis of the cell.

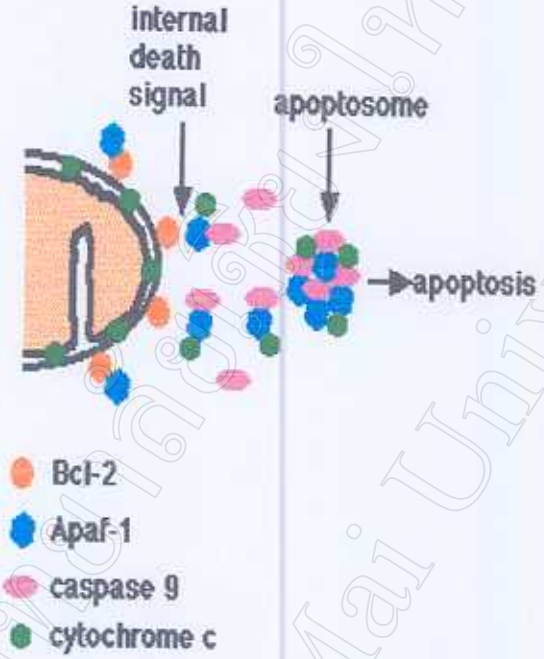

Figure 5. Apoptosis triggered by internal signals.

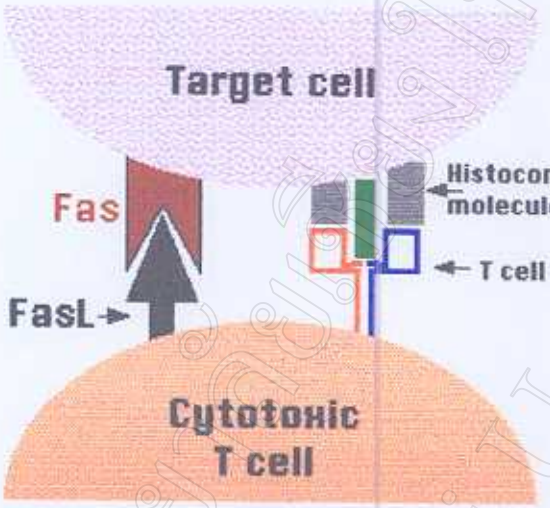

Figure 6. Apoptosis triggered by external signals

2. **Apoptosis triggered by external signals**

Fas and the TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. Binding of the complementary death activator (FasL and TNF respectively) transmits a signal to the cytoplasm that leads to activation of caspase 8. Caspase 8 (like caspase 9) initiates a cascade of caspase activation leading to phagocytosis of the cell.

The death of cells invariably takes place by a process called apoptosis. This is a programmed physiological process by which cells commit suicide in response to damage or the presence or absence of specific environmental stimuli, for example, depletion of nutrients. The Birmingham scientists have already characterised several stresses that trigger apoptosis in large-scale bioreactors.

They have also shown that ectopic expression of the anti-apoptotic cellular protein known as Bcl-2 inhibits apoptosis in NS0 myeloma, CHO and hybridoma cell lines. Bcl-2 can promote survival at very low dilution rate in continuous culture. In general, the group has found that by introducing anti-apoptotic genes into cells they can increase the robustness and survival of cells in culture.

1.2.8 Thalassemia and antioxidant

1.2.8.1 Primary antioxidant defenses

The antioxidant activity of several natural and synthetic compounds is encompassed by redox transition involving the donation of a single electron (or H atom, equivalent to donation of an electron and H^+) to a free radical species. During the course of this electron transfer, the radical character is transferred to the antioxidant, yielding the antioxidant-derived radical. Wardman P, 1988 has provided unambiguous evidence that the production of thyl radicals in biological systems

depends as much on kinetic factors as it does on the thermodynamics of individual steps or equilibria. The kinetic control of electron transfers of the type illustrated in reaction, is central to understanding the basis of antioxidant activity and requires careful consideration of the decay pathways of the antioxidant-derived radical, $A^{\bullet}$.

$$AH + X^{\bullet} \longrightarrow A^{\bullet} + XH$$

The examples of antioxidant were vitamin E, ascorbic acid, ubiquinol, uric acid and thiols.

1.2.8.2 **Secondary antioxidant defenses**

Secondary defense may be considered as a varied host of enzymes, many of which participate in metabolic systems in response to oxidant challenge and/or injury and they serve as a repair system to eliminate molecules or cell components that were damaged by oxidants or free radical reaction which escaped the primary antioxidants defense. Thus, the enzymes considered as secondary defense contribute to the repair of membrane phospholipids, proteins, and DNA. Digestion of critically damaged RNA and proteins represents their actual repair process; repair of phospholipids and DNA involves removal of oxidized portion and followed by lysophospholipid reacylation or proper base replacement, respectively

An early event resulting from oxidative injury to membrane phospholipids is the information of fatty acid acyl hydroperoxides. The disruptive effects of these apparently trigger a lipid hydrolytic activity, which could be considered the onset of the repair sequence consisting on removal of the LOOH (Van Kuijk, *et al.*, 1987). This sequence entails following steps: first, hydrolysis of the phospholipid

hydroperoxide by phospholipase A_2 to yield a free fatty acid hydroperoxide and lysophospholipid in the membrane is reacylated through the normal acyl-CoA/acyltransferase reactions which is stimulated during membrane oxidant stress (Van Kuijk, *et al.*, 1987; Lubin BH and Kuypers FA, 1991; Sevanian, 1991; Pacifici EHK, *et al.*, 1993). Thus it appears that two enzymes are central to membrane phospholipid repair, phospholipase A_2 and fatty acyl transferases.

Oxidative damage to nucleic acids occurs *in vivo* (Ames BN, 1990) with estimates as high as one base modification per 130,000 bases in nuclear DNA (Richter *et al.*, 1988) and per 8,000 bases in mitochondrial DNA; furthermore, the fragments of oxidized mitochondrial DNA have been implicated in cancer and aging (Richter C, 1988). This damage disrupts transcription, translation, and DNA replication and also gives rise to mutations. There is evidence that DNA repair enzyme activity-requiring excision of the damaged strand followed by a endogenous-polymerases-mediated insertions of the proper nucleotides- is induced oxidative damage (Howard-Flanders P, 1981; Lindhal T, 1987; Teebor GW, *et al.*, 1988). In mammalian systems, N-glycosylase activities eliminate the damaged base generating apurinic/apyrimidinic sites; mammalian redoxyendonuclease has a broader substrate specificity and remove pyrimidine base damage products *via* an *N*-glycosylase activity followed by an ATP lyase activity catalyzing α β, δ-delimitation reaction which results in removal of the deoxyribose and generation of a strand scission product (Doetsch PW, 1991; Demple B and Levin JD, 1991).

1.2.9 **Curcumin**

Curcumin (diferuloylmethane) is a major yellow pigment that has been isolated from the ground rhizome of the *Curcuma* species, Zingiberaceae (Table 1). Seven major species of *Curcuma longa* Linn., *C.xanthorrhiza* Roxb., *C. wenyujin* (Y.H. Chen et C, Ling): *C. sichuanensis*; *C. kwangsiensis*; *C. aeruginosa* Roxb.; and *C. elata* Roxb. have been cultivated in China and their composition of curcumanoids were analyzed (Chen CM and Fang HC, 1977). Three major curcuminoids namely curcumin, demethoxycurcumin and bisdemethoxycurcumin naturally occur in these *Curcuma* species. The contents of curcuminoids of these plants vary with the site and cultivation period as illustrated in Table 1. It seems that C. *longa* L. (turmeric) has the highest concentration of curcumin as compared to the other species.

Turmeric is widely used as a spice and coloring agent in several foods. Curcumin has been demonstrated to have potent antioxidant (Kunchandy E and Rao MNA, 1990; Subramanian M, *et al.*, 1994; Sreejayan and Rao MNA, 1994) and anti-inflammatory activity (Huang MT, *et al.*, 1988,1991,1997; Shin CA and Lin JK, 1993), and inhibit the carcinogen-DNA adduct (Conney AH, *et al.*, 1991) and tumorigenesis in several animal models (Huang MT, *et al.*, 1992, 1994, 1995; Rao CV, *et al.*, 1995) as shown by the findings summarized in Table 2.

1.2.9.1 **Biological activities of curcumin in *Vitro* and In *Vivo***

1.Scavenging of reactive oxygen species (ROS)

Curcumin is a potent scavenger of a variety of ROS, including superoxide anion (Kunchady E and Rao MNA, 1990), hydroxyl radical, singlet oxygen (Subramanian M, *et al.*, 1994), nitric oxide and peroxynitrite. Curcumin has the ability to protect lipids, hemoglobin, and DNA against oxidative degradation. Pure

curcumin has more potent superoxide anion scavenging activity than demethoxycurcumin or bisdemethoxycurcumin (Kunchady E and Rao MNA, 1990). Curcumin is also a potent inhibitor of ROS-generating enzyme cyclooxygenase and lipoxygenase in mouse epidermis (Huang MT, *et al.*, 1991)

2. **Inhibition of chemical carcinogenesis**

Curcumin inhibited chemical carcinogenesis in different tissue in several experimental; animal models as indicated by Table 2. Curcumin inhibited tumor initiation by benzo (a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA) in mouse epidermis (Conney AH, *et al.*, 1991). Topical application of curcumin strongly inhibited tumor promotion in the skin of DMBA-initiated mice (Huang MT, *et al.*, 1988, 1992, and 1995). Including 0.5%-2.0% curcumin in the diet decreased BaP-induced forestomach tumors per mouse by 51%-53% when it was administered during the initiation period and by 47%-67% when it was administered during the postinitiation period (Huang MT, *et al.*, 1994). Including curcumin in the diet decreased the number of N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)-induced duodenal tumors in mouse (Huang MT, *et al.*, 1994). Administration of curcumin in the diet decreased the number of azoxymethane (AOM)-induced colon tumors in mice (Huang MT, *et al.*, 1994) and in rats (Rao CV, *et al.*, 1995).

3. **Induction of apoptosis**

The curcumin (30 μM) induces apoptosis in several tumor cell lines (Jiang MC, *et al.*, 1996). The curcumin-induced apoptosis is highly dependent on the origin and malignancy of cell lines. It appears that the typical apoptosis can only be induced in immortalized mouse embryo fibroblast NIM 3T3, erbB2 oncogene-transformed NIH 3T3, mouse Sarcoma 180, human colon cancer cell HT29, human kidney cancer

cell 293, and human hepatocellular carcinoma HepG2 cells but not in primary cultures of mouse embryonic fibroblast C3H 10T1/2, rat embryonic fibroblast or human foreskin fibroblast cells (Jiang MC, *et al.*, 1996). Treatment of NIH 3T3 cells with the PKC inhibitor staurosporine, the tyrosine kinase inhibitor quinacrine induces typical apoptosis. The blocking the cellular signal transduction in immortalized or transformed cells might trigger the induction of apoptosis.

Some evidence demonstrated that curcumin (3.5 μg/ml) induces apoptosis of human promyelocytic HL-60 cells. The apoptosis inducing activity of curcumin occurred in a dose- and time-dependent manner. Flow cytometric analysis showed that the hypodiploid DNA peak of propidium iodide-stained nuclei appeared 4 hours after treatment with 7 μg/ml curcumin.The apoptotic effect of curcumin was not affected by cycloheximide, actinomycin D, EGTA, W7 (calmodulin inhibitor), sodium orthovanadate, or genistein whereas an endonuclease inhibitor, ZnSO4, and a proteinase inhibitor, N-tosyl-L-lysinechloro-methyl-ketone (TLCK), could markedly abrogate curcumin-induced apoptosis. The antioxidants N-acetyl-L-cysteine (NAC), L-ascorbic acid, α-tocopherol, catalase and superoxide dismutase all effectively prevented curcumin-induced apoptosis. Furthermore, over expression of Bcl-2 in HL-60 cells delayed the entry of curcumin-treated cells into apoptosis, suggesting that Bcl-2 plays an important role in the early stage of curcumin-triggered apoptotic cell death (Kuo ML, *et al.*, 1996).

Table 1. Curcuminoid Contents in the Rhizome of Curcuma Species

Curcuma species	Origin (year)[a]	Curcuminoid (%)[b]			
		Total	Cur	Dcur	Bdcur
Curcuma Longa Linn.	Nan-Chang (1965)	1.41	0.70	0.35	0.25
Curcuma Longa Linn.	Cheng-du (1979)	3.83	2.03	1.12	0.82
Curcuma wenyujin (Y.H. Chen et C. Ling)	Che-Chiang (1979)	0.20	0.13	0.07	0.02
Curcuma Longa Linn.	Kwang-Chou (1979)	1.28	0.63	0.40	0.52
Curcuma xanthorrhiza Roxb.	Kwang-Chou (1980)	2.10	1.43	0.86	0.12
Curcuma Longa Linn.	Beijing (1980)	3.82	1.79	1.11	0.75
Curcuma sichuanesis	Si-Chuan (1980)	0.04	0.01	0.01	< 0.01
Curcuma Kwangsinensis	Yun-nan (1980)	1.54	0.89	0.57	0.23
Curcuma aeruginosa Roxb.	Si-Chuan (1980)	0.04	0.01	0.01	< 0.01
Curcuma elata Roxb.	Kwang-see (1980)	0.01	< 0.01	< 0.01	< 0.01
Curcuma Longa Linn.	Nan-ning (1981)	3.97	1.84	1.09	1.01

Source: Chen and Fang (1997)

[a]origin, the site of cultivation or collection; year, the time of sample collection.

[b]Total, total curcuminoid; Cur, curcumin; Dcur, demethoxycurcumin; Bdcur, bisdemethoxycurcumin.

Table 2. Biochemical Actions of Curcumin

Biochemical action	Reference
Inhibits TPA-induced ornithine decarboxylase (ODC) mRNA and activity	Huang et al. (1988)
Scavenges superoxide anion and hydroxyl radical	Kunchandy and Rao (1990)
Inhibits lipoxygenase and cyclooxygenase activity	Huang et al. (1991)
Inhibits arachidonic acid metabolism	Conney et al. (1991)
Inhibits the formation of carcinogen-DNA adducts	Conney et al. (1991)
Inhibits skin tumor initiation and promotion	Huang et al.. (1992)
Inhibits TPA-induced cellular 8-hydroxydeoxyguanosine	Shin and Lin (1993)
Scavenges singlet oxygen	Subramanian et al.(1994)
Inhibits lipid peroxidation	Sreejayan (1994)
Inhibits BaP induced forestomach and lung tumorigenesis	Huang et al. (1994)
Inhibits ENNG-induced duodenal tumorigenesis	Huang et al. (1994)
Azoxymethane-induced colon tumorigenesis in mice and rats	Rao et al. (1995)
Inhibits TPA-induced skin inflammation	Huang et al.(1997)

1.3 Objective

1.3.1 To elucidate apoptosis in immature red blood cells and peripheral blood mononuclear cells of β-thalassemic patients.

1.3.2 To study correlation of anemia and apoptosis in patients

1.3.3 To study the effect of oxidative stress to apoptosis.

1.3.4 To study the effect of antioxidant to inhibit oxidative stress and apoptosis.