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Caspase : Leads to Apoptosis using VHDL and SPICE

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Abstract: A family of cystein-dependent aspartate-directed proteases, called caspases, is responsible for the proteolytic cleavage of cellular proteins leading to the characteristic apoptotic features, e.g. cleavage of caspase-activated DNase resulting in internucleosomal DNA fragmentation. Currently, two pathways for activating caspases have been studied in detail. One starts with ligation of a death ligand to its transmembrane death receptor, followed by recruitment and activation of caspases in the death-inducing signaling complex. The second pathway involves the participation of mitochondria, which release caspase-activating proteins into the cytosol, thereby forming the apoptosome where caspases will bind and become activated. In addition, two other apoptotic pathways are emerging: endoplasmic reticulum stress-induced apoptosis and caspase-independent apoptosis. The model for cell death has been implemented using Very High Speed Integrated Circuit Hardware Description Language (VHDL) programming language (Xilinx Tool), and PSPICE taking three input signals: Tumor necrosis factor-? (TNF), Epidermal growth factor (EGF) and Insulin

Keywords: Apoptosis, Caspase, Tumor Necrosis Factor-α, Epidermal Growth Factor, Insulin.

1. INTRODUCTION

Death belongs to life as well as birth; whenever there is a new life born, another life ends somewhere. There is a time and place for all things and appropriate death brings the scales into balance, not only on the scale of the global ecosystem, but also within a single living organism [1, 2, 3]. If there is too much or not enough death, the life of the whole organism is endangered. Actually, the presence of "meaningful" death is crucial to the very existence of multicellular organisms; the organism as a whole benefits from the controlled removal of certain individual cells that are unnecessary or faulty. Active cellular suicide, apoptosis, [4, 5, 6, 7] plays an important role in the correct formation of the nervous system during development. Furthermore, during neurodegenerative conditions, such as Alzheimer's disease [8, 9], apoptosis may have a major impact on the outcome. In neuronal cells, the constant interaction between pro-apoptotic and pro-survival molecules and signaling pathways is considered to determine whether the cell will survive or not.

Thus, apoptosis [10, 11, 12] is an active process; to precede it needs activation and synthesis of many signaling and effectors molecules. Although the final outcome of the apoptotic process is always the same, the actual molecular mechanisms are diverse and many of them still remain to be elucidated. The traditionally described characteristics of apoptotic cell death include chromatin condensation, fragmentation of DNA, blebbing of the plasma membrane, and formation of "apoptotic bodies", which are phagocytes by neighboring cells.

If the pro-apoptotic signaling exceeds the strength of the signal signifying pro-survival, the cell is likely to undergo apoptosis. However, to be able to undergo apoptosis, the cell should complete a highly regulated

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process that requires new RNA and protein synthesis and the activation of specific, normally dormant cellular enzymatic machinery. The term apoptosis/ programmed cell death was coined in the early 1970 [4, 5, 6], although the phenomenon of apoptosis was described already in the late 1700s. The word, apoptosis, is derived from Greek roots meaning, "Dropping off" e.g. falling of leaves. It plays an important role in the development and maintenance of tissue homeostasis but also represents an effective mechanism by which abnormal cells, such as tumor cells, can be eliminated. Abnormalities in apoptotic function or resistance to apoptosis have been identified as important events in the pathogenesis of colorectal cancer and its resistance to chemotherapeutic drugs and radiotherapy.

2. BIOLOGY OF APOPTOSIS

Almost all cells, regardless of their phylogenetic origin [12, 13, 14, 15] or physiological specialization, ultimately sense and die. There are several ways of dying, depending on the nature and severity of the death stimulus, type of the cell affected, and homeostatic conditions of the cell and its surroundings. The two major forms of cell death recognized today are apoptosis and necrosis [4, 16, 17, 18]. The main difference is thought to be the requirement for energy: apoptosis is an active process consuming energy and requiring macromolecular synthesis, while necrosis occurs passively. In spite the fact that there are several features that can distinguish these processes from each other, also common and overlapping characteristics appear, and there are cases where no clear cut distinction between apoptosis and necrosis can be found. While the characterization of differences between apoptotic and necrotic cell death remains incomplete shown in Table 1, recent findings suggest that apoptotic cell death differs even from cell to cell, and each induction strategy is likely to involve a unique set of genes [16, 17].

Table 1
Programmed Cell Death Pathways

APOPTOSIS	*NECROSIS*
Caspase activation inhibition of mRNA translation	Pro-inflammatory signaling and cytokine production
Condensation of cell and organelles	Swelling of the cell and organelles
Chromatin condensation DNA fragmentation	Mottled chromatin condensation
Loss of membrane asymmetry	Loss of membrane asymmetry
Membrane remains impermeable	Rapid loss of membrane permeability
Cell falls apart into apoptotic bodies	Cell membrane explodes.
	Remains stay together

Role of caspases/ interleukin-1b-converting enzymes (ICE): Caspases, [19, 20, 21] the family of proteases largely responsible for the execution phase of apoptosis. The caspases are present in cells in an inactive form (pro-caspases) and form a tightly regulated, sequential, and self-amplifying cascade. Caspases are responible for almost all the biochemical and morphologic features of apoptosis and act by the proteolytic cleavage of a host of cellular proteins. All caspases share a number of structural and functional features, two of which are reflected in the name caspase itself. The "c" refers to the fact that caspases are cysteine proteases, with the catalytic site cysteine contained within a conserved QACXG motif (single letter amino acid code), whereas the "aspase" refers to the unique and absolute predilection of all caspases for cleaving proteins after aspartic acid residues. While is not yet possible to provide a complete flow diagram of the precise cascade of reactions by which caspases mediate the apoptotic pathway, caspases can be divided into two main functional classes; "*initiator*" caspases and "*effector*" caspases. Caspase-mediated celavage of apoptosis-specific endonuclease CAD (caspase-activated DNase) is responsible for the "ladder" pattern of DNA fragmentation typical of apoptosis. Caspases also are responsible for proteolysis of the nuclear lamins therby facilitating nuclear condensation. Caspases that target cytoskeletal proteins such as á-fodrin, â-actin, and keratins mediate dissasembly of the cell cytoskeleton. Other classes of caspase substrates cleaved during apoptosis include DNA repair enzymes, signal transduction molecules, as well as transcriptional and cell cycle regulators.

There are currently 14 members of the caspase (cysteinyl aspartate-specific proteinases) family [19]. They sequence identity with CED-3, a protein, required for developmental cell death in the nematode ditis elegans[19]. It is evident that caspase play a critical role in many biochemical events controlling in nematodes and mammals. This conclusion is based on several observations: first, caspase hydrolyze that are known to be selectively cleaved at the beginning of apoptosis: lamins, inhibitors of caspase-ivated DNAse (DFF45/ICAD), PARP [22]. Secondly, post-translational activation of several caspases relates with the main apoptotic events: plasma membrane blebbing, shrinkage of the cytoplasm, dilation of oplasmic reticulum, nuclear chromatin condensation and DNA fragmentation [23]. Thirdly, naturally occurring e inhibitors such as viral proteins Crm A (cytokine response modifier A) and p35 as well as the IAP (the r of apoptosis) family of proteins and Bcl-2/Bcl-xL (inhibitors of cell death, induced by many stimuli) all revent apoptosis. Finally, mice deficient in caspase-3 suffer a severe defect in apoptosis during brain velopment.

The family of caspases can be divided into three subfamilies based on their biological functions: the ICE subfamily (caspases-1,-4,-5), which activate cytokines during inflammation, initiators of apoptosis (caspases-2, -8, -9, -10) and executioners of apoptosis (caspases-3,-6, -7).

All caspases have four amino acids [2, 3, 4, 12] in their substrate cleavage site and aspartic acid in the P1 position. Caspases are synthesised as inactive proenzymes, comprising an N-terminal peptide (prodomain), one large and one small subunit, and they contain in their structure a conserved pentapeptide active-site motif with the catalytic cysteine. The difference in structure of various proenzymes may be due to the length of the prodomain and the presence of a linkage peptide between the two subunits.

3. DOWNSTREAM SUBSTRATES OF CASPASES

Once activated, apoptosis activator caspases such as caspase-2, -8 and/or -10 will activate other downstream apoptosis executioner caspases including caspase-3, -6, and -7 [24]. Furthermore, active caspase 8 can cleave Bid to tBid, which translocates to the mitochondrial membrane and triggers cytochrome c release and activation of the mitochondrial apoptotic pathway. The activated executioner caspases can subsequently cleave distinct cellular proteins such as PARP [poly(ADP-ribose) polymerase], lamin, fodrin, and also Bcl-2, leading to the characteristic morphological changes (Figure 2).

3.1 Caspase-3, caspase-6 and caspase-7

Caspase-3, a key factor in apoptosis execution, is the active form of procaspase-3. The latter can be activated by caspase-3, caspase-8, caspase-9, caspase-10, CPP32 activating protease, granzyme B (Gran B), and others. The downstream substrates of caspase-3 include procaspase-3, procaspase-6, procaspase-9, DNA-PK, PKCã, PARP, D4-GDI (D4 GDP-dissociation inhibitor), steroid response element-binding protein, U1-70kD, inhibitor of caspase activated deoxyribonucleic (ICAD) and so on [25, 26]. Except for á-fodrin and top isomerase's I, all of the substrates can be cleaved during the apoptosis in caspase-3"/" cells, from which we can see that caspase-3 is not the only apoptosis executioner caspase. Through alternative splicing, caspase-3 pre-mRNA can be translated into a short caspase-3 (caspase-3S), which lacks the conservative 'QACXG' sequence in the catalyzing site, and is co-expressed with caspase-3 in all human tissues. Caspase-6 and caspase-7 are highly homologous to caspase-3. Procaspase-6 can be activated by caspase-3 but not Gran B. Caspase-6 can also activate procaspase-3 by a positive feedback pathway. The substrates of caspase-6 include PARP, lamin and procaspase-3. Procaspase-7, whose substrates include PARP, procaspase-6 and steroid response element-binding protein, can be activated by Gran B.

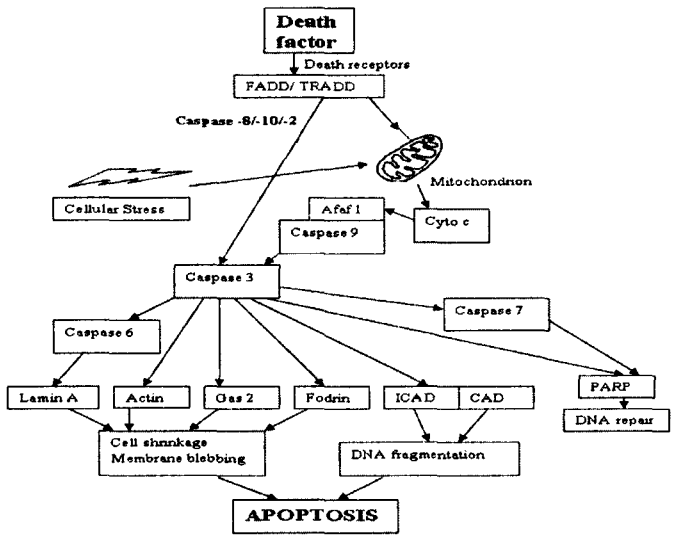

Figure 2: Downstream Substrates of Apoptosis Executioner Caspases Apaf-1, Apoptotic Protease Activation Factor-1; CAD, Caspase-activated Deoxyribonuclease; FADD, Fas-associated Death Domain; ICAD, Inhibitor of Caspaseactivated Deoxyribonuclease; PARP, Poly(ADP-ribose) Polymerase; TRADD, Tumour Necrosis Factor Receptor-associated Death Domain.

3.2 Capase 9

Caspase-9 (ICE-LAP6, Mch6) is a member of the CED-3 subfamily and bears similarity to Caspase-3, though there is a main difference in the active-site pentapeptide: Caspase-9 shows the sequence QACGG instead of the more usual QACRG [27]. Compared to Caspase-3, Caspase-9 possesses a longer N-terminal prodomain with high similarity to the prodomains of CED-3 and Caspase-2, which contain CARDs. Caspase-9 is ubiquitous expressed, with high levels of mRNA expression in the heart, testis and ovary. Multiple mRNA species are found, due to alternative splicing. In cytoplasmic extracts of HeLa cells, Caspase-3 is activated in a strictly dATP-dependent manner by the cooperative action of three protein factors, designated apoptotic protease activating factors (Afaf's) While Apaf-1 contains a sequence homologous to the C.elegans CED-4, and while Apaf-2 was identified as Cytochrome c, Apaf-3 was shown to be identical with Caspase-9. It also was demonstrated that, in presence of dATP, Caspase-9 is directly activated by Apaf-1 and Cytochrome c [27]. Active Caspase-9 activates Caspase-3 and by this the apoptotic machinery that leads to DNA fragmentation and cell death.

Regulation of Caspase-9 by phosphorylation: Interestingly, it has been reported that Caspase-9 activity is regulated by phosphorylation the kinase Akt phosphorylates Pro-Caspase-9 at Ser196 and by this inhibits proteolytic processing of pro-Caspase-9. Akt itself is activated by the phosphatidylinositol 3-kinase (PI3K) pathway which is positively controlled by Ras and negatively by the PTEN tumor suppressor.

3.3 Caspase-2

Caspase-2 is the earliest identified caspase in mammals. This enzyme is unique for its features of both initiator and effector caspases [4, 5, 6]. Caspase-2 appears to be necessary for the onset of apoptosis triggered by several insults, including DNA damage, administration of TNF, and different pathogens and viruses [19]. Both caspase-2 and caspase-9 are similar to CED-3 in *C. elegans*, all of them with a CARD. Caspase-2 widely distributes in most tissues and cell types. It can be found in the nucleus as well as the cytoplasm, with a considerable portion in the Golgi complex. Many studies have shown that caspase-2 serves as an apoptosis inducer in some types of cells. The complex formed through the recruitment was enough to activate procaspase-2. In this case, procaspase-2 might be activated upstream of procaspase-9 activation, the release of cytochrome c and other apoptosis factors inside the mitochondria. The release of cytochrome c was not accompanied by any obvious alteration in nuclear pores. Only in the late phase of apoptosis, caspase-2 entered the cytosol because of an increase in the diffusion limits of the nuclear pores. Furthermore, caspase-2 could induce the release of cytochrome c, AIF and second mitochondrial activators of caspases/direct IAP binding protein with low pI (Smac/DIABLO) from mitochondria, independent of Bid or other cytosolic factors. Mitochondrial cytochrome c released by caspase-2 was sufficient to

activate apoptosome *in vitro*. In 2002 it was found that in caspase-2-deficient cells, the translocation of Bax from the cytosol to mitochondria, induced by etoposide, was inhibited.

3.4 Caspase-12 and Endoplasmic Reticulum (ER) Stress-induced Apoptosis

Caspase-1, caspase-4, caspase-5, caspase-11 and caspase-12 are highly homologous. Caspase-12 localizes in ER and mediates apoptosis under ER stress. It plays a key role in many nervous systems diseases, such as Alzheimer's disease. ER stress is mainly caused by the accumulation of proteins, particularly unfolded and malfolded ones, in ER lumen and/or the perturbation of calcium ion homeostasis. Thapsigargin, tunicamycin, calcium ionophores, brefeldin-A and cisplatin can all induce ER stress [4, 5, 6]. It has been proved in some cell types that ER stress can lead to apoptosis in which caspase-12 is involved. In apoptosis caused by tunicamycin, the processing of procaspase-12 at its N-terminus was necessary not only for the translocation of active caspase-12 into the nucleus but also for the apoptosis. Under ER stress, the activation of procaspase-12 could be induced by other caspases. The stress inducers can lead to the translocation of caspase-7 from the cytosol to the ER surface. Caspase-7 activates procaspase-12 by exsecting its prodomain through interaction. This activation manner may be employed in all prolonged apoptosis caused by ER stress. The functions of mitochondria in this type of apoptosis varied with different reports. The activated caspase-12 then activates procaspase-9, and the activated caspase-9 in turn activates procaspase-3, -6 and -7 (Figure 2). In these newlyfound caspase-activation pathways, no cytochrome c was found to be released from mitochondria, which implies that cytochrome c is not involved in the activation of procaspase-9, and, in this case, procaspase-9 is the downstream substrate of caspase-12.

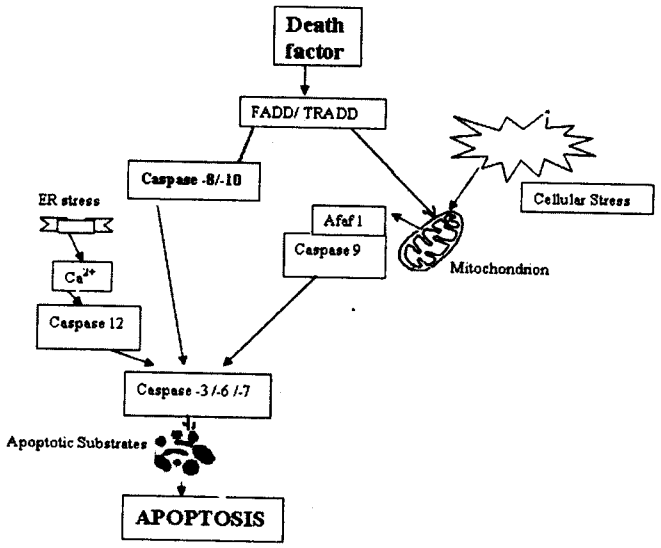

Figure 2: Caspase-12 Involved in Apoptosis Triggered by ER Stress Apaf-1, Apoptotic Protease Activation Factor-1; ER, Endoplasmic Reticulum; FADD, Fas-associated Death Domain; TRADD, Tumour Necrosis Factor Receptor-associated Death Domain.

4. MECHANISMS OF CASPASE ACTIVATION

In general, there are several ways by which caspases can be activated. The **first** is as a result of cleavage by autolysis and by upstream proteases, such as other caspases or granzyme B (GrB), which is the only mammalian serine protease that shares the caspase specificity for the Asp residue at the P1 position. A common feature of the ICE family proteases is the presence of a prodomain that has been hypothesized to keep the enzyme in an inactive form [28, 29, 30]. Prodomain is necessary also for dimerization, which occurs prior to auto processing. GrB in vivo processes initially caspase-3, which in turn can remove the prodomain from caspase-7, and, finally, caspase-7 is fully processed by GrB. In a heterologous expression system it has been shown that recombinant caspase proenzymes are autolytically processed to their mature forms when synthesized at sufficiently high levels. A mutation in catalytic cysteine residue prevents this processing. Isolated caspase procnzymes can be auto activated when they are concentrated by ultra filtration. In this study we are not using this mechanism.

A **Second mechanism/ Extrinsic pathway** of caspase activation is through death-inducing receptors of tumour necrosis factor receptor family (TNFR/NGFR), including Fas/CD95/Apo1, TNFR1, TNFR2, DR3/W 1/Tramp, DR4/TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor-1), DR5/TRAIL-R2/TRICK2/Kil and DR6. These receptors contain both cysteine-rich extracellular domains and intracellular cytoplasmic dea domain (DD). The interaction of the appropriate ligand with the receptor results in its trimerization and recruitme of adapter molecules [43, 44, 45]. In the case of Fas-induced apoptosis, adapter protein FADD (Fas-associate death domain or Mort 1) contains C-terminal DD, which promote the interaction with the same DD in trimeriz Fas-receptor, and N-terminal DED (death effector domain), which associates with DED in the large N-termin peptides of caspase-8 (Figure. 3). This complex is called DISC (death-inducing signalling complex), and it thought that as more procaspase-8 molecules become involved in this complex, they start to be activated, probabl by autocleavage. Similar mechanisms have been demonstrated for the tumour necrosis factor receptor (TNF-R1 which seems to require an additional adapter molecule TRADD (TNFR-associated death domain), which recrui FADD and procaspase-8. Furthermore, TRADD can recruit serine-threonine kinase RIP (receptor interactin protein) and an adapter molecule RAIDD, which has sequence similarity with the prodomains of caspases-2, and CED-3 and can activate procaspase-2. At the same time, the physiological role of caspase-2 in the activatio cascade of caspases remains unclear. It is likely that caspases-8 and -10 are the major apical proteases in TN and anti-Fas induced apoptosis, which activate the downstream caspases. In the next step, activated downstrean caspases can activate themselves and other caspases, leading to the amplification cascade of caspase activation The recruitment of caspases-8 and -10 may be inhibited by viral inhibitors of the apoptotic signals (FLIPs). Thes inhibitors contain two N-terminal DEDs in their structure. They interfere with FADD-caspase-8 binding and inhib both caspase-8 activation and apoptosis. Caspases -1, -2, -4, -5, -9 and CED-3 contain another "caspas recruitment domain" (CARD), required for assembly of activation complexes.

The **third mechanism/ Intrinsic pathway** in which the caspase cascade can be initiated involves translocatio of cytochrome c from the mitochondria to the cytoplasm (non-receptor-mediated pathway or stress-induce apoptosis) (Figure 3). The mechanism of this translocation remains unclear and may be due to opening of mitochondrial permeability transition pore, rupture of the outer membrane or the presence of specific channels fo cytochrome c. Cytochrome c release from mitochondria is under the control of the Bcl-2 family of proteins, tha either inhibit (Bcl-2, Bcl-xL) or promote (Bax, Bak, Bik, Bid) apoptosis. In the cytoplasm, cytochrome c interacts with Apaf-1 (the human homolog of C. elegans protein CED-4).

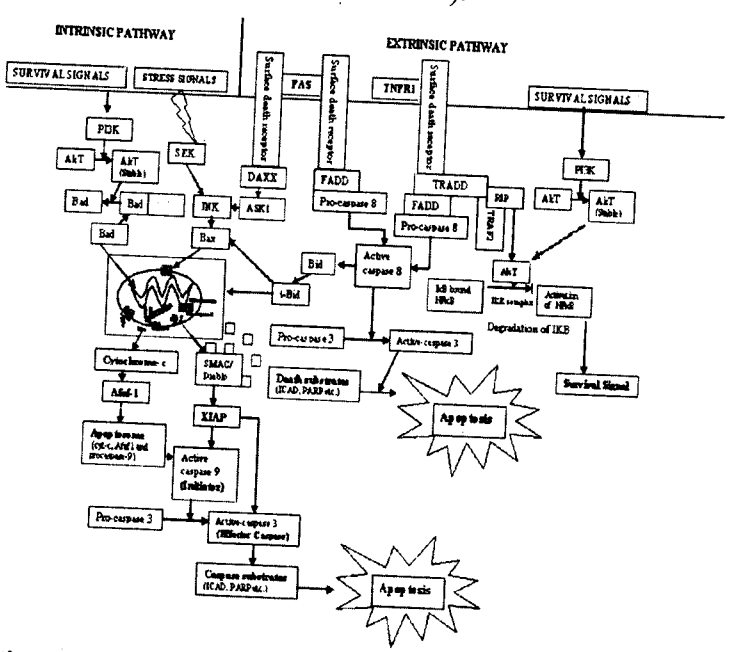

Figure 3: Schematic Representing the Core Components of Apoptosis Pathways. In the Extrinsic Pathway, TNF Super Family members Including Fas Ligands Binding to a Death Receptor and Forming a Death Inducing signalling Complex (DISC), Which Activate Caspase-8. In the Intrinsic Pathway, Cytochrome c released from Mitochondria Causes Apoptosome Formation and Caspase-9 Activation. Both Caspase-8 and Caspase-9 Activate Down Stream Caspases like Caspase 3 and Leading to Apoptosis.

5. RESULTS AND DISCUSSIONS

Based on the pathways discussed we had made truth tables for every possible path for cell death. Than we realize the truth tables by Karnaugh Map (K-Map) and get the Boolean expression for its individual possible paths. We simulate the results of each path, then combine all the results, and simulate through SPICE and VHDL for prediction of cell death.

5.1 Using Very Large Scale Integration (VLSI)

This is the field, which involves packing more and more logic devices into smaller and smaller areas. VLSI has opened up a big opportunity to do things that were not possible before. VLSI has been around for a long time, there is nothing new about it, but as a side effect of advances in the world of computers, there has been a dramatic proliferation of tools that can be used to design VLSI circuits. For implementing truth table we have written code in VHDL and with the help of XILINX tool we simulate the results and get the output as shown in Figure 4.

For cell survival the ten different proteins i.e. P13K, TNFR1, EGFR, IRS, IKK, Grb2, SOS, Ras, TRADD, Traf2 should present. Figure 4 shows the output signal of cell survival/ death from XILINX simulator considering TNF, EGF and Insulin as input. In figure a[0:9] shows the input for ten proteins which we have mentioned above that should be '1' always for cell survival. b[0:2], c[0:2], d[0:1], e[0:2], and f[0:2] shows the different pathways related to TNF, EGF and Insulin. b[0:2] means this pathway is three input and all inputs are in form of '0' or '1'. k, l, m, n, and o shows the outputs of all possible pathways.

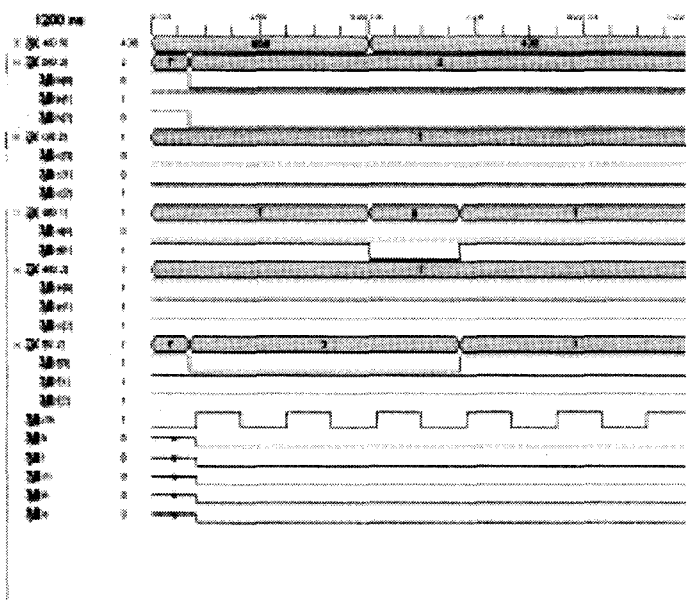

Figure 4: Output using Xilinx Tool

5.2 Simulation Program with Integrated Circuit Emphasis (SPICE)

It is a general-purpose analog eletronic circuit simulator. It is a powerful program that is used in IC and board-level design to check the integrity of circuit designs and to predict circuit behavior. Semiconductor devices such as diodes, transistors and integrated circuits can be found everywhere in our daily lives, in Walkman, televisions, automobiles, washing machines and computers. We have come to rely on them and increasingly have come to expect higher performance at lower cost.

Digital logic is a rational process for making simple "true" or "false" decisions based on the rules of Boolean algebra. "True" can be represented by a 1 and "false" by a 0, and in logic circuits the numerals appear as signals of two different voltages. Logic circuits are used to make specific true-false decisions based on the presence of multiple true-false signals at the inputs. The signals may be generated by mechanical switches or by solid-state transducers. Once the input signal has been accepted and conditioned (to remove unwanted electrical signals, or "noise"), it is processed by the digital logic circuits. The various families of digital logic devices, usually integrated circuits, perform a variety of logic functions through logic gates, including "OR", "AND", and "NOT", and

combinations of these (such as "NOR", which includes both OR and NOT). One widely used logic family is TTL (transistor-transistor logic). Another family is CMOS (complementary metal oxide semiconductor logic), which performs similar functions, but consumes less power.

5. 2.1 USING Complementary Metal Oxide Semiconductor (CMOS)

CMOS was also sometimes referred to as complementary-symmetry metal–oxide–semiconductor (or COS-MOS). The words "complementary-symmetry" refer to the fact that the typical digital design style with CMOS uses complementary and symmetrical pairs of p type and n type metal oxide semiconductor field effect transistors (MOSFETs) for logic functions.

Two important characteristics of CMOS devices are *high noise immunity* and *low static power consumption.* Significant power is only drawn when the transistors in the CMOS device are switching between on and off states. Consequently, CMOS devices do not produce as much waste heat as other forms of logic, for example transistor transistor logic (TTL) or NMOS logic, which uses all n-channel devices without p-channel devices. CMOS also allows a high density of logic functions on a chip. "CMOS" refers to both a particular style of digital circuitry design, and the family of processes used to implement that circuitry on integrated circuits (chips). CMOS circuitry dissipates less power when static, and is denser than other implementations having the same functionality. As this advantage has grown and become more important, CMOS processes and variants have come to dominate, so that the vast majority of modern integrated circuit manufacturing is on CMOS processes. We get output using CMOS (shown in Figure 5). We simulate the results of each path, then combine all the results, and simulate through SPICE simulator using CMOS, get result of TNF, EGF and Insulin for its cell survival/death. In output, '1' signifies cell survival and '0' signifies cell death. For cell survival the ten different proteins i.e. P13K, TNFR1, EGFR, IRS, IKK, Grb2, SOS, Ras, TRADD, Traf2 should present. If any one of them is absent than there is a cell death. In Figure 6 first three blocks shows the input and last block show the output of TNF. V(2), V(3) etc represents the output of each possible path used for TNF, EGf and Insulin as cell survival/ death. We get final output from V(53).

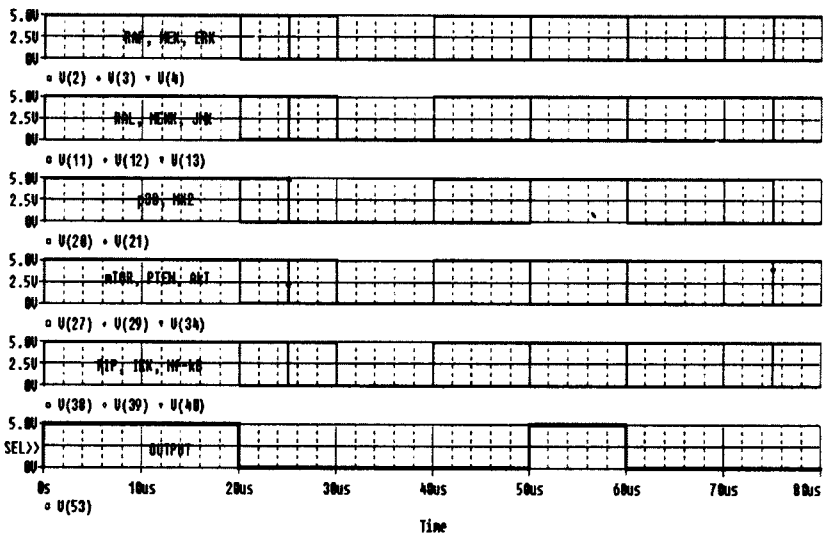

Figure 5 : Output using CMOS

6. CONCLUSION

We have demonstrated that the VHDL programming language, and SPICE can be applied to predict the cell death with a high level of accuracy using input TNF, EGF and Insulin. The signaling pathway has reproduced experimental data with accurate. Understanding the nature of signaling networks that control the cell death is very significant and theoretical calculations, in particular the simulation process developed using SPICE and VHDL, seen to be a proper tool for gaining such understanding. The results obtain will give information on how the input signals inducing cell death should be modulated to achieve desire outputs and thus helps the experimentalists to design proposals regarding possible improvements to cell death.

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