Cell and Molecular Biology
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Week 03
Cellular Homeostasis
Lecture - 14
Apoptosis and Autophagy

Hello everyone. This is Dr. Vishal Trivedi from the Department of Biosciences and Bioengineering, IIT Guwahati. In this module, we are discussing the different aspects related to cell growth and regulations. So far, what we have discussed is the importance of growth and the significance of growth in the context of prokaryotic and eukaryotic cells, and how growth is regulated by having a well-developed cellular system. We have discussed these regulations in the prokaryotic system as well as the eukaryotic system; subsequently, we have also discussed the different types of growth media that you can use for the prokaryotic or eukaryotic system.

And then later on, in the previous lecture, we also discussed the different stages of cell growth. So where we have discussed mitosis as well as meiosis. And we have also discussed the different types of methods, how you can monitor these events in the experimental system. In today's lecture, we are going to discuss what will happen when the cell is devoid of growth or media and how it will actually respond to adverse conditions for proliferation.

So now when we talk about growth, the cell is actually going to require growth. The cell requires growth, and growth is linked to the production of energy. Or you actually require a lot of energy for, you know, driving those reactions to produce the energy. And the energy is linked to nutrition. Right.

So that's why we have discussed different types of cell culture media, how they actually provide the various nutrients needed to synthesize different biomolecules, including DNA, proteins, nucleic acids, and so on. Now nutrients are so there could be, you know, the condition where there will be a scarcity of nutrition. So when the cell enters a stage where it is actually going to experience starvation or where there will be no nutrition. Right. Then it will enter a stage of starvation.

Now, during the starvation, it is actually going to start using the reserved food material. So when I say reserved food material, these are actually the materials that are being produced when there is an excess amount of nutrition. So what happened when you were taking the nutrition is that it was going to be divided into two parts. One part that is going to be utilized for producing energy so that you can perform growth and run different types of metabolic pathways. On the other hand, if nutrition is access to the amount of energy that you are utilizing for growth and other kinds of biological processes, then this nutrition is going to be converted into reserve food material, like, for example, fat.

So it is actually going to get converted into one of the reserve materials, which is called fat. It can also be converted into another food result, which is called glycogen, right? Although we are not discussing cellular metabolism, but rather the nutritional means of carbohydrates, right? Nutrition is mostly carbohydrates and fats, which are actually utilized for producing energy, right? With the help of the different types of catabolic pathways, such as glycolysis, the Krebs cycle, and those for carbohydrates. And then for the fat, it is going to undergo beta oxidation. And subsequent to that, it's going to have an electron transport chain, right? So if there is an excess amount of glucose, then glucose is going to form glycogen. And if there is an excess amount of fat, then it is actually going to be converted into fat.

Now, once you enter a stage of starvation, glycogen will be converted into glucose. And then glucose is again going to be utilized to produce energy. Similarly, the fat that is stored will be converted into fatty acids, and the fatty acids will also be utilized during starvation. Now, remember that this food reserve material is limited. Right? You are actually going to have a limited quantity of food reserves.

So once the food reserves are depleted, right? Or it's not going to be presented. Then the cell will enter into acute starvation. Then it is actually going to enter into acute starvation, or it is not able to sustain the required amount of energy needed for running even the basic metabolic pathways; for example, you require at least the energy for running your lungs. You require the energy for running your kidneys. You require energy at least for running your heart, right? Then only you can be able to survive.

But if you have not been able to even provide the energy for running these, you know, the life-saving processes, then you will enter into acute starvation, and after acute starvation, the cell will decide whether it can sustain life or whether it should go and die. So after this, it will enter the death pathway. Right? Or it will enter the death pathway. Now when it enters the death pathway, it is actually going to have two different modes of entering the death pathway. One is called the programmed death pathway, and the other is called non-programmed or necrosis.

This is being called programmed to program within the program. You can have two different types of pathways. You can have apoptosis. And you can also have autophagy. OK, both apoptosis and autophagy are programmed.

That's why they are not going to damage the cell or the body much. Whereas necrosis, which occurs in a non-programmed fashion, actually damages the cells or the particular organ. So in today's lecture, we are going to discuss the death pathway and how death, if it follows the programmed pathway, actually occurs in apoptosis and autophagy, and if it is non-programmed, what other events happen in necrosis. So what is meant by apoptosis? Apoptosis means programmed cell death. Its opposite, or the antonym, is called necrosis.

So whatever we are actually controlling in the organism, that is apoptosis. And programmed cell death can be of two types. It could be the one that is apoptosis. The

other one is called autophagy. Apoptosis is happening within the cytoplasm, which is actually where you are going to start eating your own organs.

and the formation of large vacuoles that eat away at the organelles before the nucleus is destroyed. So remember that in the cell, you have multiple copies of mitochondria, multiple copies of lysosomes, multiple copies of the Golgi, and so on. Right. So you probably have the 500 copies of mitochondria. So if you can take or eat some of those copies, you will probably be able to recycle the material and sustain yourself for a certain amount of time, especially when you are under acute starvation conditions.

And for apoptosis, it is actually going to, you know, have a set of reactions that are actually going to drive the programmed death of that particular cell. So apoptosis is a Greek word that means falling off or dropping off. It is a self-destruction program genetically sequenced in the biomedical events. It is a kind of counterbalance through the elimination of a simple, similar number of cells. And it has basically been called death by design.

So when you talk about apoptosis, you can also discuss the differences between apoptosis and necrosis, because only then will you understand why apoptosis is beneficial for the organism and why necrosis is not beneficial. So when you talk about the differences, apoptosis is a programmed cell death. So it's an energy-dependent programmed cell death, whereas in the case of necrosis, it is where the cell dies due to damage. So if any cell undergoes different types of injuries and if the injury is beyond a limit where repairing is not possible, then it will enter into necrosis. Apoptosis may occur in one cell.

Necrosis may occur in a group of cells. Apoptosis does not lead to inflammation, whereas necrosis is very, very important and is actually responsible for the production of inflammatory reactions. And that's why it is going to damage more tissue, because when you have the inflammatory reactions, that is all I think we are going to discuss when we talk about molecular immunology in the later part of this particular course. You will see that the inflammation is going to produce a large amount of ROS, a large amount of free radicals, a large amount of nitrogen center radicals, oxygen center radicals, and so on. And that is actually going to damage the neighboring tissues.

So it is actually going to damage a larger amount of tissue than what is actually being injured. Then there is a central role of mitochondria in regulating programmed cell death, whereas there's no role of mitochondria in the case of necrosis. Then, in the case of apoptosis, the dead cells are going to be eaten by the macrophages. So it is actually going to be cleared off very systematically. Whereas in the case of necrosis, the dead cells are removed by macrophages.

So apoptosis is the process where the cell goes through and shrinks. Whereas in the case of necrosis, the cell is going to swell, which means the cell is actually going to have a greater amount of inflammatory molecules. And that's why the necrosis is actually called an unhealthy mode of death. Whereas apoptosis is actually a programmed cell death. So,

in apoptosis, what will happen is that you have a normal cell, correct? And then this normal cell, when it actually, you know, is going to start catalyzing the events of apoptosis, is going to enter into cell shrinkage.

And there will be chromatin condensation. After that, the membrane is going to be distorted, and then there will be membrane blobbing, which means it's going to form a blob-like structure. And then after this, you are actually going to have the nuclear content, which is going to collapse. And then ultimately, it is going to form apoptotic bodies. And then these apoptotic bodies are actually going to be engulfed by the macrophages.

There are many players who are going to participate in the apoptosis. So you can have the apoptosis-inducing factors. You're going to have different types of caspases. You're going to have the Bcl proteins.

You're going to have an apoptosome. And then you're also going to have the p53 gene as well as the p53 proteins. Now talking about the caspases, these are cysteine proteases, and they are specific proteases that have been catalyzing a cascade of reactions to drive apoptosis. It is a family of 16 proteases that are present in the cell in their inactive form. So they are actually going to be present as the inactive forms, and the inactive forms are actually going to be cleaved at one end. And that's why it is actually going to be converted into the active voice.

So you're going to have the, you know, like. You're going to have it like this, right? So this is the inactive form. And when there is a signal, right? That signal is actually going to cleave off this particular bond. And that's how you are going to have it, right? And this is going to be the active form. And this active form is going to be a cysteine protease.

And the job of this active form would be that it is actually going to activate another caspase, and that's why you are actually going to have two classes of caspases. One is called the initiator caspases, which are actually going to initiate the reactions, and the second one is called the effector caspases, which are actually going to affect the cells. So in the initiator caspases, you are going to have caspase 2, 8, 9, and 10, whereas in the effector caspases, you are going to have caspase 3, 6, and 7. An initiator caspase is going to take up the signal, like the death signal, and then it is actually going to catalyze the different types of reactions, whereas the effector caspases are actually going to directly affect the biomolecules. For example, in the case of caspase 3, it is actually going to start degrading different types of proteins and different types of DNA.

So it is actually going to activate the DNAs, and then the DNAs are going to start cleaving the DNA part. So these are the classes of different types of caspases, and this is the structure of the caspases. So you are going to see that the majority of these caspases are actually participating in apoptosis. Some of these caspases are also involved in the inflammatory pathway and so on. So caspases actually have two different types of domains.

One is called the caspase recruitment domain or the CARDs. And the other one is called the debt effector domain. So, these are called debts. Now, this is what I have already explained. You are going to have the active caspases, and when the active caspase is formed, it will actually initiate the cleavage reactions for the other caspases.

And that's how it is actually going to catalyze a cascade of reactions. Then the second molecule is called the Bcl2 family. So they are the regulatory proteins of the mitochondria. And it is the first time a gene has been isolated from B cell lymphoma, which is a cancer. And that's why these proteins are being called Bcl2.

They all have the BH3 domain like the Bcl-2 domain. And BH3 is a pro-apoptotic domain that gets exposed and activated. Now, when talking about the Bcl2 family, you're going to have BH1-4, BH1-3, and BH3. And within the BH1-4, you're going to have the anti-apoptotic proteins, which are called Bcl2 and Bcl-XL. Whereas in BH1-3, you're going to have the pro-apoptotic proteins, which are called Bax, Box, and Bak.

And then you have BH3, which is referred to as a pro-apoptotic protein, namely Bad, Bik, and Bid. And what is the function of the Bcl-2 family? It is regulating the balance between pro-apoptotic and anti-apoptotic situations. It is involved in the intrinsic pathway, and it is going to induce the VCL2 via the death signal. So Bcl2, which is called Bcl lymphoma, BH, which is called Bcl2 homologs, Bid, which is called Bcl2 interacting domain, BcXL, which is called X or the close homolog, and the L, which is going to inhibit apoptosis. So Bcl-2 and Bcl-2XL, right? Then we have the apoptosome, which is a multi-unit protease activation complex that forms after the induction of cell death through the mitochondrial pathway.

This discovery of the apoptosome is being made by Professor Wang, and it is a wheel-like structure connected to seven radial spikes. Now, talking about the machinery, we are going to discuss the pathways that are important for apoptosis. So you have two different types of pathways. One is called the death receptor pathway, which is known as the extrinsic pathway. And then you also have the mitochondrial pathway, which is called the intrinsic pathway.

In the intrinsic pathway, the inducers are from the cell. The extrinsic pathway is induced from outside. So this is actually the place where the distinction lies. So you don't have to remember which reaction is going to induce the extrinsic pathway or the intrinsic pathway. In the majority of the cases, if the inside of the cell is not performing well, it is actually going to induce the intrinsic pathway.

If it is getting any kind of threat from outside, then it is mostly going to initiate the extrinsic pathway. For example, if you are treating a cell with a drug, right? then that it is actually going to activate the extrinsic pathway. But if you are treating with the drug and the drug is going inside and is actually changing the microenvironment, then it is actually going to induce the extrinsic pathway. Now let's first talk about the death receptor pathway. So induction is mediated to the cell surface receptors known as death receptors.

So basically, on the cell, you actually have the receptors, and some of these receptors are going to respond to the drug molecule, and that's how it is actually going to initiate the cascade of reactions related to the death receptor pathway. Death ligands are activating these receptors and the further activation of caspases is going to lead to cell death. So whatever machinery we have discussed for apoptosis is going to be downstream of these initiator pathways. Whether it is a death receptor pathway or the mitochondrial pathway, the initial induction is going to be different.

Inside the cellular machinery, it is going to be the same. It is going to use the caspases. The Bcl2 proteins are going to be used. It is going to use the apoptosome, and so on. Right. Now, within the death receptors, you are actually going to have, you know, different types of death receptors.

So this one of such receptors is the TNFR superfamily, where you have the death receptors like TNFR1, CD95, TRAIL R1, and TRAIL R2, and these are their corresponding ligands. So which one is called TNF alpha, and the other one is called CD95L or Fas ligand? Then you also have the TRAIL R1, and these are the full forms of the 10FR and all that. And what happens ideally is that the ligand actually binds to the receptor. So once there is an induction of that receptor pathway, the ligand, which is TNF alpha in this case, will go and bind to the death receptor, which is present on the plasma membrane. So, you see the plasma membrane, right? And so, the binding of the ligand to the death receptor is going to initiate the downstream signaling.

And this downstream signaling is actually going to activate the apoptotic pathway. And that's how these are actually going to be the extrinsic pathway. So you have the different types of receptors: the fast ligands, fast receptors, DR4, DR5, TNFR1, DR3, and all that. So these are actually going to be the different types of receptors, and they are actually going to participate in the different types of reactions. Once the receptor binds its ligand, it will actually bind the adapter proteins, the different types of adapter proteins.

For example, most of these are going to bind to the FADD, and then these adapter proteins are actually going to drive the downstream signaling, and ultimately, they are actually going to collect the cellular machinery for apoptosis. So they are going to activate caspase 8, caspase 10, and so on, and then ultimately it is going to form the apoptosome, and that's how it is actually going to damage all the proteins, DNA, and so on, and ultimately these cells are going to form the apoptosomes, and these apoptosomes are actually going to be taken up by the macrophages. So this is exactly what it is showing here in the extrinsic pathway. You are actually going to bind the ligand, and then the ligand is actually going to, you know, take up the adaptor proteins, and then these adaptor proteins are going to activate the inactive caspases, like procaspase 8 and 10. Into the active caspase 8 and 10, and then these active caspases are going to activate the downstream effector caspases like caspase 3, 6, and 7, and then ultimately it is going to activate the production of the apoptosome, and that's how it is going to induce apoptosis.

Now, coming to the mitochondrial pathway. So it is going to be the release of the protein

from the mitochondrial immune membrane into the cytosol. So the inducer is that the proteins from the mitochondria are going to be released into the cytosol. Right. And then it is going to induce DNA damage. And what are the inducers for the mitochondrial pathway? So you're going to have cellular stress, heat shocks, oxidative stress, and cellular damage, which are actually inducers of apoptosis that follow the mitochondrial pathway.

So what happened exactly is that you are going to have cell damage, right? And you are saying that cell damage is going to activate the p53, which is actually going to activate the pro-apoptotic Bcl2, and then pro-apoptotic ClL2 is going to induce the release of mitochondrial factors into the cytosol, such as cytochrome C. Once cytochrome C is released, it is going to form a complex with the upper one. And that's how it is actually going to activate caspase 9, so procaspase 9 is going to be activated and form caspase 9, and that's all these are going to come together and form the apoptosome, and these apoptosomes are then going to initiate a cascade of caspase activations. And that's how it is going to activate caspase 3, and the caspase 3 activation is ultimately going to lead to the damage of the filler machine.

So this is exactly what is going to happen. The apoptosome is going to form. Then it is going to activate caspase 9. Caspase 9 is going to activate caspase 3. And then once caspase 3 is activated, it will induce apoptosis.

So this is exactly what is going to happen. You have healthy cells; they are going to get an injury. If the injury is moderate and repairable, then the cell will enter this phase and go to get the healthy cells. If the injury is beyond repair, then it is causing different types of genetic disorders. You know, it will not enter, it will not reverse, right? It will not go back, right? And then it is also possible that you got injured the first time, then you got another additional injury, and that's how, if the cell decides that it will not be repairable, right? Then it will initiate the caspase activation, and it is going to initiate programmed cell death, and that's how the cell is going to enter apoptosis. So, a damaged cell may undergo apoptosis if it is unable to repair the genetic disorders or genetic errors.

Now, what is the significance of apoptosis? So, the significance is that it is actually going to delete the dangerous and damaged cells. It is required for the development of the organs. Without apoptosis, humans can grow. The whole epithelial cell lining changes every 23 days. So basically, apoptosis is actually regulating cell growth, so it is not allowing cell growth to go beyond a limit, and that's how it keeps killing the cells.

Then it is also required for the regulation of immune cells such as T cells, and it is also required for the development of cancer and neurodegenerative diseases. Although we are not discussing in detail how apoptosis relates to the development of cancer or neurodegenerative diseases, we can discuss that later in some other courses. Now, talking about the pathological implications: the growth of tissue and organs in the embryo, the replacement of time-expired cells like the gut epithelium, repair and healing after injury or inflammation, and the regeneration of tissue and hyperplasia. So these are some of the events where apoptosis is taking part actively; if apoptosis is not present, it will actually

cause a more pathological impact.

Then you also have the hepatic implications. So in the new generator for diseases like Alzheimer's disease, apoptosis is being induced in the neurons, and that's how there will be neural damage. And when there is neural damage, it will actually reduce the number of neurons. And that's how it is actually going to be responsible for the development of diseases like Alzheimer's. Then we also have the myocardial infarctions.

So myocardial infarction happens when there is a development of hypoxia. And because of the hypoxia, the stem cells are probably not getting enough oxygen. And that is going to induce the production of ROS. And because of the ROS production, the stem cells are going to be damaged in the heart. And that's how it is actually going to cause the myocardial infarction. Then we also have, you know, some of the apoptotic cells or apoptotic proteins as targets for anti-cancer drug development because if you can induce apoptosis in cancer cells, you can induce apoptosis because it is controlled by two different types of proteins: one is the pro-protein and the other is the anti-protein, right? So you have pro-apoptotic proteins or you have anti-apoptotic proteins.

Now, pro-apoptotic proteins are going to increase apoptosis. Anti-apoptotic proteins are going to decrease apoptosis. So if you increase apoptosis, you are actually going to, you know, enter or be able to damage the cells. If you have the anti-apoptotic factor, then it is actually going to be responsible for the development of cancer because these cells will survive. And since these cells are already being damaged genetically, there is a highly likely chance that these cells may enter into cancer. So if you increase the anti-apoptotic factors, like some of the Bcl2 proteins, then it is actually going to allow the cancer cells to survive.

And because of that, it is not good for them. So some of these cells probably can be used as anti-cancer drugs. Then we also have neurodegenerative diseases. So you can have Alzheimer's disease, which shows excessive apoptosis. Then you have neural growth factors and brain-derived neurotropic factors. Then you secrete the protein that maintains the balance between the pro-apoptotic and the anti-apoptotic factors.

And then the blockage of these factors is a new era of interest for the target drug WRE. Then we also have the myocardial infarctions. So it occurs due to the blockage of the coronary artery by the thrombus. Right. And the decrease in oxygen supply due to necrosis or apoptosis. So you can actually have cell death through two pathways, which are called necrosis and apoptosis.

Then you can also use apoptosis to target the cancer cells. So you can actually use the BCL-2 proteins as targets for new drug development. BCL-2 is anti-apoptotic and increases resistance to cancer chemotherapy, and the death receptors and the respective guidelines can be used in targeting anti-cancer drugs. Now how are you going to detect apoptosis in a particular type of cell? So you actually have multiple ways in which you can detect apoptosis in cells. So you can actually use the detection of programmed cell death either by morphology, or you can use molecular assays such as DNA

fragmentations, flow cytometry, and enzyme assays.

Detection of the morphology. You know that the classical morphology of apoptosis is that there will be an induction of cellular shrinkage. Then you also have the detachment from its neighbors. For example, you know the cells are growing like this, don't you? They are tied to each other. But you can have the cells like this.

If you have a cell like this, that means it is actually detaching from its matrix. And that is the clear indication that probably something is happening with this. Then you can also have fragmentation of the nucleus. You will see that the nucleus is like this, right? Ideally, you will see a nucleus like this. So this is a healthy nucleus.

When you see the nucleus like this, it means that it is actually inducing apoptosis. And then you can also have the packaging of the cell into multiple plasma membranes, which means you're actually going to have a cell like this. You're going to have to like this. Those are the apoptotic cells. And then you're going to have phagocytosis of these fragments.

So these cells are going to fragment. Then you can also have the detection of DNA fragmentation. So remember that when I think of the subsequent lecture, we are going to discuss the genome packaging. Right. So when you talk about genome packaging, right? So the genome is actually forming a nucleosome. Right. So you remember that you are actually going to have different types of nucleosomes and the DNA is unwinding on this and then going like this.

Right. Like this. Right? So DNA is like this. Right? And here you have the H1. Right? This is a linker histone. So when you are exposing this genome to the enzyme, what enzymes are doing is cutting this.

They are cutting from here. Right. They are cutting in between. Right. And because of that, the length that is being covered or wrapped onto the nucleosome is going to be released.

Right. And this is actually going to come into the fragments. Right. So these fragments would be like X. Then, once you get from here, you're going to get 2x; then, you get from here, it's going to be 3x, right? And because of that, it is actually going to give you a pattern which is called the DNA ladder. So this has been called a DNA ladder, although we don't have enough time to discuss the process and all those kinds of things. But when you isolate the genomic DNA from a damaged cell and resolve it onto the agarose, you will get a DNA ladder like this because all these fragments are separated from one another by the length of this particular DNA. And because of that, it's going to form a ladder, right? So you're going to have x, 2x, 3x, 4x, 5x, like that, right? So basically, it is going to keep increasing, right? So remember that this length is 180 base pairs, right? So it's going to be 180 for this one; it is going to be 360 for this one; it is going to be like that, right? So when agarose gel is used to separate this genomic DNA, you will obtain DNA fragments and DNA ladders.

And this particular assay is called the DNA fragmentation assay. And the application of the total cellular DNA to an agarose, the fragments are separated throughout the agarose gel, and the fragments will be detected by the ETBR staining. Then you can also use flow cytometry. Remember that apoptosis is going to have a lot of changes in the cell.

It is actually going to change the nucleus. It is going to change the outer plasma membrane, and so on. And that is all we have not discussed. So flow cytometry can be used to detect these modified cells. So what you can do is use flow cytometry, stain the cells with different types of dyes, and then analyze them using flow cytometry. And we have prepared a small demo clip where the students are actually going to explain to you how you can stain the cells with different types of dyes that will stain the nucleus as well as the plasma membrane, and that's how you can detect apoptosis. Hello everyone, in this video, we will discuss how to perform live dead cell staining using acridine orange and propidium iodide on fat cells.

So the basic principle is that the acridine orange is permeable to both live and dead cells, whereas propidium iodide is only permeable to late apoptotic and necrotic cells. So this property of acridine orange and propidium iodide lets us recognize what population of cells are in late-apoptotic, early-apoptotic, or necrotic states. So, coming to the procedure, the first thing we do is trypsinize the cells from the 100 mm cell culture dish and then plate 1 million each in the completed and diluted wells. So after 2 to 14 hours of adherence, we treat the samples according to our requirements, and then let's say that we are treating for 24 to 48 hours; after the appropriate time, we trypsinize the cells, collect the pellet, wash it twice with previous solution, and then re-suspend the pellet in 2% fetal bovine serum in phosphate buffer saline. So after we have resuspended the pellet in 2% FBS in phosphate-buffered saline, we give the appropriate acridine orange and propidium iodide treatment.

The working concentration for acridine orange is 0.5 to 1 microgram/ml, whereas the working concentration for propidium iodide is 1 to 5 micrograms/ml. So we add the dyes just before taking the data, or we can just give 10 to 15 minutes of incubation for the dyes to bind to the cells, and after that, we acquire the data on the Cellquest Pro. So after adding the acridine orange and propidium iodide to the cells, we have to acquire the data on the CellQuest Pro software. The first thing we do is open the CellQuest Pro and connect it to the cytometer, and then we need the counters, the detector, the amps, and the status for the acridine orange and propidium iodide staining. We need two dot plots; one is for the FSC and SSC for the forward scattering and the side scattering, and the other one is for the FL1 and FL3.

The FL1 plot is on the x-axis, whereas the FL3 plot is on the y-axis. The FL1 plot is for acridine orange, and the FL3 plot is for propidium iodide. After taking the plots, we have to set the directory and save the data in our required location. In the detector and AMS, we have to remember that we must set the population of the healthy cells in the first quadrant, which is 10^1 and 10^4. After we set the untreated cells in the first quadrant, we analyze the treated cells and then can say whether there is any shift in the fluorescence

between the untreated and treated cells.

For the treated cells in the third and fourth quadrants that represent the apoptotic and necrotic cells. So now we will be taking the sample, but before analyzing the data, we have to set the number of events to 5000 and then keep it on setup. First, we will see whether the events are coming properly or not. Now we press acquire, as we can see that in the FSC plot and SSC plot, we can see the events coming near 0, 0, which represents the healthy population. Additionally, in FL1 and FL3, we have set the healthy population between 10^1 and 10^4, so this represents the first quadrant.

We will show in detail how to do the quadrant analysis in the FCS-5 software. Now that we have set the population in the first quadrant, we will remove it from setup and acquire the data. After the untreated samples, we have to take the treated samples on the same parameter description that we have set for the untreated cells. Now we change the sample in the sample injection port to the treated sample. We have to remember that we don't have to change the parameters, or else we will never be able to say whether there is any shift in the untreated or the treated cells if we change the parameters. After changing the sample, we now have to choose the directory for the treated cells, then change the name to "treated," set the file count to one, and press OK; then we can acquire the data using the same parameters.

As we can see, there is some shift in the population of the cells; the population is having a little bit more fluorescence than the untreated cells, which represent the apoptotic and necrotic cells in the third and fourth quadrants. In the fourth quadrant, mostly the necrotic and dead cells are present, whereas in the third quadrant, the late apoptotic cells are present. After we take the untreated and treated samples, after we acquire the data for the untreated and treated samples, we have to analyze the data in the FCS 5 Express Pro software using quadrant analysis. In the quadrant analysis, we can see how many populations of cells are present in each quadrant, and therefore we can identify the number of healthy populations, the apoprotein, and the necrotic cells.

So after acquiring the data in the FACS equipment, we now have to analyze the data in the FCS using Access software. So the first thing we do is open the new layout and change the orientation to landscape. And now we input the data. The first thing we do is take the untreated file and then open the dot plot. We need two dot plots.

The one is the FSC SSE and the other one is the FL1 FL3; the FL1 FL3 dot plot shows the live and dead cell staining. The FL1 is responsible for the ethylene orange, whereas FL3 is responsible for the propidium iodide. And now we take the treated file, and again we select the dot plot; in the dot plot, we also need the FSE and the FL1 FL3 plot. As we can see, there is a difference between the untreated and the treated samples. Now we have to find out what percentage of the cells have actually undergone apoptosis or necrosis.

We have to divide the population of cells into four quadrants using the quadrants option. So we go to the gating, then take the quadrant, and apply it to the FL1, FL3 plot. We have to apply it in such a way that we cover all the cells in the untreated plot, so let's say that

in the untreated plot we have 92% live cells and seven percent are in the early apoptotic phase. And then, after applying the quadrant to the untreated, we have to apply the same quadrant to the treated one in order to find out the difference between the two. So, we just click on the quadrant and then we copy and paste it onto the treated one.

In this way, we can say that in the first quadrant, in the untreated sample, we have approximately 93% live cells. Whereas in the treated one, we have only 35.7% healthy. Whereas 33% are in the late apoptotic phase and 29% are necrotic cells. So, in this way, we can use acridine orange and propidium iodide.

To determine the healthy, apoptotic, and necrotic cells in different treatments. We can also establish a relation between different concentrations of treatment and the number of live and dead cells in any experiment. So this is the way we analyze and process the data on the FACTS equipment in order to perform the live and dead cell screening. So I hope you can understand the different processes or events that are required for studying apoptosis using flow cytometry. And it may be useful for you to understand the process, as well as to be able to use this process in your laboratory. Now, let's talk about another program of cell death, which is called autophagy, where the cell is going to start eating its own organelles.

Now, autophagy literally means—auto means self and phagy means eating, right? So when you start eating your own self, then it is called autophagy, a process by which the cell breaks down and destroys old, damaged, or abnormal proteins and other substances in its cytoplasm. The breakdown products are then recycled for important functions, especially during periods of stress or starvation. So mostly it happens during this starvation. When you remove the essential amino acids and the large quantity of glucose, the cell actually requires those essential amino acids. So what it is going to do is start taking the proteins from the cytoplasm, or it is going to start taking the different organelles such as mitochondria, Golgi body, lysosomes, and so on, and then it's going to take up.

So these are some of the inducers for autophagy. One is nutrient starvation. The second one is a response to the hypoxia. The third one is called ER stress. The fourth is called cell death. The fourth and fifth are called cell damage, and the sixth is called oxidative. Although these inducers are different, the downstream molecular events are going to be the same, right? The majority of these molecules, or the majority of these inducers, are actually going to initiate a particular cascade of reactions, and because of that, they have actually induced autophagy.

Now, when you induce autophagy, it follows different pathways. So you can have micro autophagy, chaperone-mediated autophagy, and also macro autophagy. In microautophagy, some proteins and organelles are directly joining the lysosomes or late endosomes. Generally, it is a non-selective process, but in some cases, it is selective. For example, the micropexophagy, microautophagy of the nucleus, and micromitophagy.

So, in some of these cases, the organelles are going to join the lysosome directly.

Remember that the lysosome is going to have different types of proteases and also nucleases. So it is actually going to be utilized for digesting the organelle and its content. And that's how these raw materials are then again going to be distributed to the cell for its usage. Then we have chaperone-mediated autophagy. So some special cytosolic proteins have a specific guide motif, which is called KEERQ, with hsc70 and other chaperones attached to the lysosomal membrane.

That guide motif attaches them to the LAMB-Az receptor at the surface of the lysosome and transports them into the lysosome with the LAMB-A2 dependent transporters. So in chaperone-mediated autophagy, chaperones are cellular degradation machinery that are actually utilized for degrading unfolded or misfolded proteins. So basically, the proteins that are actually going to have this particular motif will be delivered to the chaperone.

And then this chaperone is actually going to deliver all these to the lysosomes for their recycling. Then we also have macrophagy. So macrophagy is where you will have the nucleation and the extensions. There will be a closer, then you are going to have fusion with the lysosomes, and then the lysosome is going to digest the material, and that's how you are actually going to have the recycling of this material. What are regulators? So there are many proteins that actually regulate autophagy, such as Bcl2, ROS, calcium, AMP-activated protein kinases, BNIP3, DRAM, Calpain, FADD, and IP_3. And we are not going to discuss their regulatory mechanism and so on, because then it is going to be beyond the scope of this particular lecture, right? One protein that we are going to discuss is called mTOR. mTOR is actually regulating the induction of a process in cooperation with the other two nutrient sensing pathways, which are protein kinase A and Sch9.

In mammalian cells, mTOR appears to regulate autophagy in a similar way to that in yeast. The mammalian target of rapamycin or mTOR acts as a negative regulator, and the extent of autophagy is regulated by the proteins upstream of mTOR signaling, including PTEN, PDK1, Akt, and TSC1/2. P10 and TSC 1/2 positively regulate autophagy, whereas Akt actually inhibits it. So it is actually going to induce apoptosis, whereas this is going to be inhibited.

So downstream of mTOR, including the elongation factor 2 and the SX kinase, which are shown to regulate autophagy. Then we also have p53. So p53 is a suppressor. It's mutated in approximately 50% of human cancers and induces autophagy. p53 can be a positive or negative regulator of autophagy depending upon its subcellular localization and the type of stress. It has been reported that the P53 function as a critical mediator for the damage-induced apoptosis has been shown to induce autophagy in a damage-regulated, autophagy-dependent manner to execute full cell death in human cancer cells.

DRAM is a lysosomal integral protein and is a direct target of hereditary-induced macrophagy and its accumulation in the autophagosomes. Diseases related to autophagy include degenerative diseases, metabolic syndromes, aging, infectious diseases, autoimmune problems, diabetes, cardiomyopathy, and cancers. Now, let's talk about what we have discussed in this particular lecture. So we have discussed the conditions under which the cell is actually going to be under extreme starvation conditions. And once it is

under extreme starvation conditions, it has two choices.

One option is that it can go to programmed cell death, or the second is that it can go to non-programmed cell death or necrosis. Within the program of cell death, we have two different modes. One is called apoptosis. The other one is called autophagy. Apoptosis is going to induce cell death, whereas autophagy is actually going to utilize the cellular machinery again and again so that the cell can overcome acute starvation. In the majority of cases, autophagy is induced first, and when even autophagy cannot sustain the cells for survival, then only apoptosis is induced, and that's how apoptosis kills the cells.

And apoptosis is going to utilize the two different types of pathways for initiating the death pathways. So, it is going to be either a death receptor pathway or the mitochondrial pathway. In the death receptor pathway, the death receptors are always present on the plasma membrane, and they actually receive the signal from the death ligands. And that's how it is actually going to initiate the activation of a series of caspases, and that's how it is actually going to induce cell death. Whereas in the intrinsic pathway, the cellular factors are released from the mitochondria under the influence of several, you know, the apoptotic inducers.

And then these mitochondrial factors are going to activate the different types of caspases. And then ultimately, caspase 3 is going to be activated, and this caspase 3 is going to kill or destroy the proteins, as well as activate the DNase, and these DNases are actually going to chew the DNA or the genomic DNA. How are you going to detect the apoptosis? So we have discussed the morphological parameters. We have discussed the DNA fragmentations, and we also discussed how you can use flow cytometry to detect apoptosis in mammalian cells. So with this brief discussion about apoptosis and autophagy, I would like to conclude my lecture here. Thank you.