Tissue Engineering
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Lecture - 17
Cell Isolation - Part 1

In the last class, we were talking about cell source right. We talked about where the cells will be obtained and what type of cells we can get and so on. Because when you are talking about tissue engineering, cells are the second aspect of the triad. During that time, I also said the cells which we get would also have to be cultured. So, there are different aspects to it. We identified what could be the source and what type of cells we could use, but we should also know how to get these cells from the tissues and how to get the specific cell type from the tissue because the tissue is going to have multiple cell types.

So, we need to be able to isolate the specific cell type which we are interested in, so that is also crucial. And once you have that, we should also look at how the cells would be cultured. And in case we are working with stem cells, how the cells would be differentiated. So, all these aspects are crucial when you are talking about cells.

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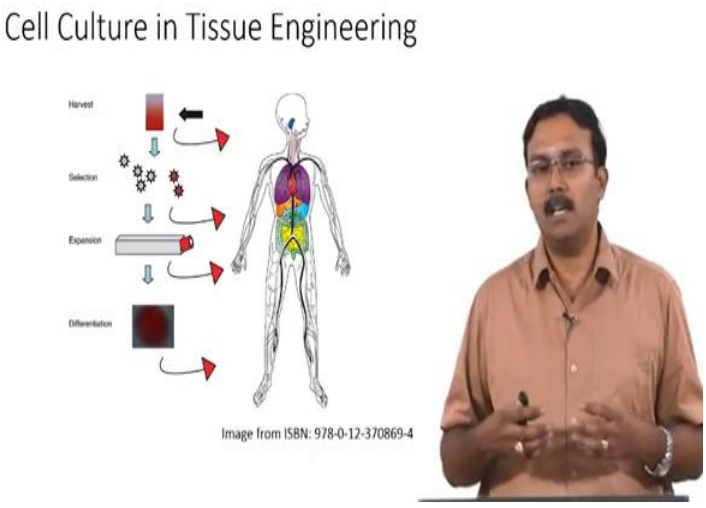

The cell culture aspect is what we are going to be talking about. We will talk about first harvesting the cells, selecting and isolating the cells, expanding or culturing the cells, and differentiating the cells. So, these are different aspects of cell culture.

Harvesting just means that taking cells from a tissue. So, it could be from different tissues. You take out the tissue of interest, and there could be some simple mechanical digestion or some simple treatment to get a mixture of cells from the harvested tissue that could be used as the cells itself, that mixture could be used as the cells itself.

One example would be just using platelet-rich plasma or platelet-poor plasma, where not much of selection or anything is done. There is a little bit of isolation being done, but it is primarily just harvesting and using it. You could also have a selection, which could be a lot more specific, where you try to identify specific cell types that you are interested in and taking those cells and using them for tissue engineering applications. It could either be in vitro, or it could be directly injected into a person for the regeneration of the tissue.

These are two options when you are talking about cell selection and harvesting. But the cells you get is usually not of enough numbers; we did talk about this earlier right. So, we need a lot of cells. And especially if you are going to take it from an autologous source, then you are not going to have enough number of cells. So, you would have to culture them. Usually, this is done in vitro so that you can get enough numbers.

Even if you are going to harvest 10^4 or 10^5 cells, you might need 10^8 or 10^{10} cells. So, you might have to multiply these cells over a period of time to get enough cells and then use it for regeneration or tissue engineering. Culturing the cells is a crucial aspect, and this is going to be dependent on the type of cell you are working with. For example, if you are working with hepatocyte, the culture conditions would be different compared to what you would have for an epithelial cell. So, you need to know what culture conditions are and how you maintain these culture conditions, what are the growth media required, and so on.

If you are using stem cells, then obviously, the expansion would also have to make sure that the stemness is maintained. So, in the sense that, as it is dividing and multiplying, it should not differentiate; it should only multiply, so that is a crucial aspect. If it starts multiplying, you will get enough numbers; then, the stem cell has to be differentiated to get the specific cell type for the application. So, the differentiation process itself will be different. How you direct the differentiation to get the desired cell type is also a challenge that needs to be understood, and this will be different for different cell types.

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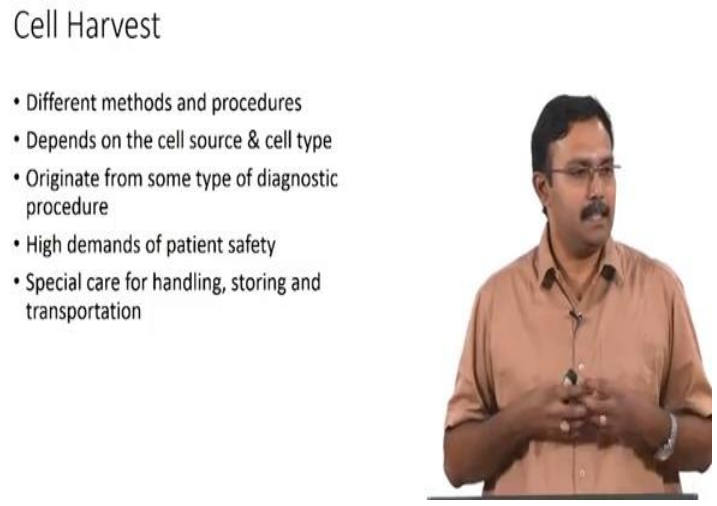

The first thing is harvesting the cells. There are different methods and procedures. So, the method you use would depend on the cell source or the cell type itself. Depending on the tissue, which you are going to extract, you are going to use different techniques. Most of these cell harvesting techniques come from some type of diagnostic procedure. There are different diagnostic procedures for studying, for checking the pathology, checking some disease condition. Here, tissues are taken for these studies. So, these techniques are used for cell harvesting, as well.

There is a high demand for patient safety because these procedures expose the patient towards potential infections, and so on. So, you need to make sure that these are done in a proper clinical environment, and you do not cause any complications to the patient. You need to maintain the correct environment, the clean environment for doing this. Special care for handling the storage and transportation of these tissues is also required. Depending on the tissue type, you might have to store it under different conditions.

You might have to store it in liquid nitrogen, or you process it immediately, or you might have to heparinize it if you are going to using blood or citrated blood might be required. Depending on what tissue you are working with and how you are going to use it in the future, you would have to handle it and store it appropriately.

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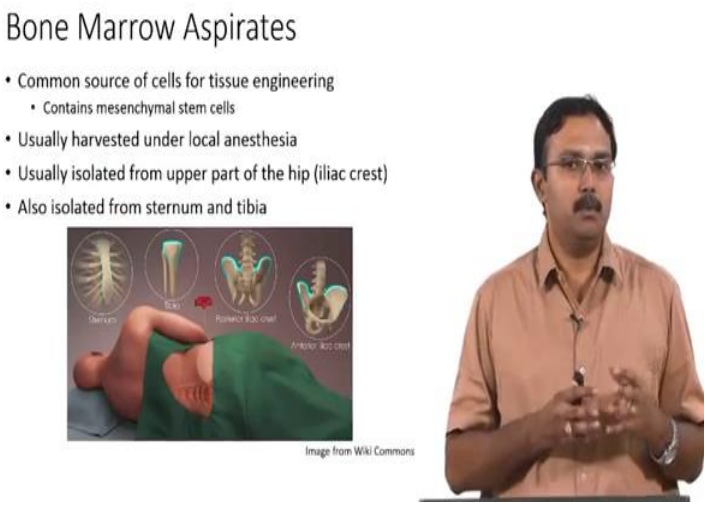

One of the most common sources for cells in tissue engineering is bone marrow aspirates because these contain the mesenchymal stem cells. These are usually harvested under local anesthesia. People are given local anesthesia, and the bone marrow aspirates are taken from the upper part of the hip, which is the iliac crest. I mentioned this earlier, as well. So, this is the iliac crest. You have the hip bone.

So, what you see here, these are the iliac crests. Here, the bone contains the bone marrow, and you can get the bone marrow aspirate from which you can get these mesenchymal stem cells. These can also be isolated from the sternum. The sternum is what is in your ribcage. The ribcage is connected by something called the sternum, so that is what this bone is. And tibia is the other bone in your legs. You can harvest mesenchymal stem cells and bone marrow aspirates from these tissues. So, the iliac crest is the most common tissue from where you try to harvest it.

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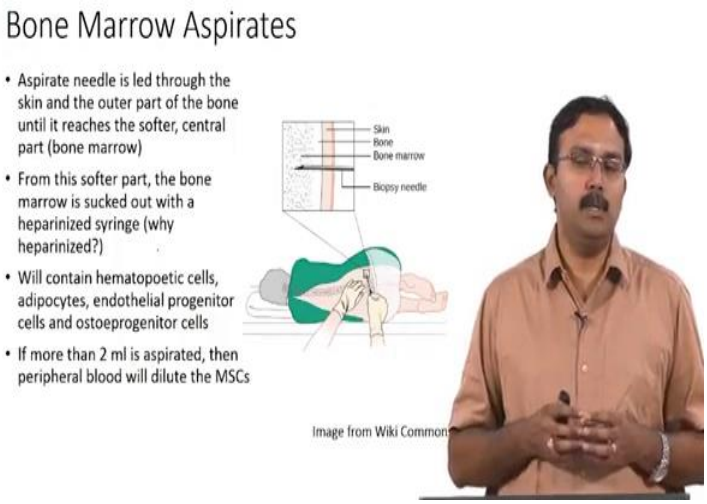

What you do is there is something called an aspirate needle. So, these are large needles, maybe 16 gauge or something. These are penetrated into the skin, and it also penetrates the outer part of the bone, which is the hard bone and reaches the softer central part, which contains the bone marrow. This is a painful procedure, so that is why you have to have local anesthesia. So, we are actually causing a puncture through the skin through the muscle tissue into the bone. So, it is going to be quite painful. And from this softer part of this bone where the bone marrow is present, the marrow is sucked out using a heparinized syringe. Why do you think we use a heparinized syringe? What is the role of heparin?

Student: To prevent blood clots.

So, these aspirates can clot very rapidly. To prevent that, you need to use a heparinized syringe. These aspirates can contain hematopoietic cells, adipocytes, endothelial progenitor cells, and osteoprogenitor cells, as well. If more than 2 ml is aspirated, then peripheral blood can dilute the mesenchymal stem cells. It is important to control how much you are aspirating, and then whatever you are getting is a now a mixture of cells, and you might have to separate cells to get the specific cell that you are looking for.

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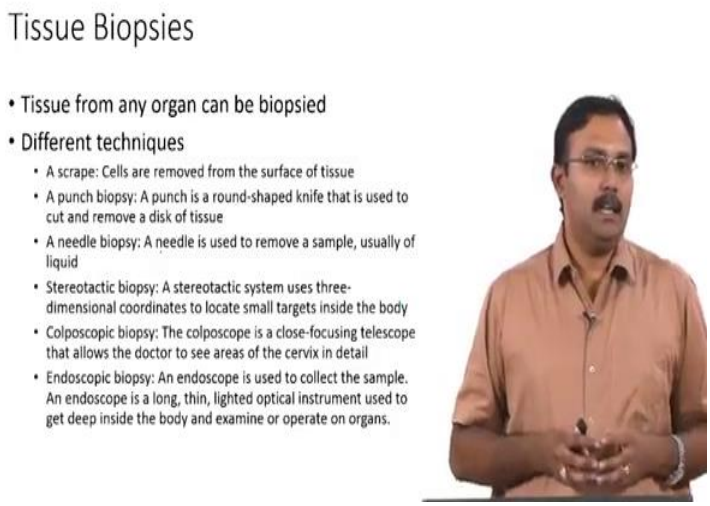

Another technique that is used is tissue biopsies. Tissues from almost any organ can be biopsied. You just take a piece of the tissue, cut a piece of the tissue, and use it for your cell isolation. So, there are different techniques for this. A scrape is basically cells are removed from the surface of a tissue, you just take a scrape of the tissue. Or you can have something called a punch biopsy where a punch which is round-shaped is used to cut and remove a disk of tissue. This is like what you would see in a punch hole. You create a small punch, and this disk-shaped tissue can be harvested, and that can be processed further to get the desired cells.

You can also have a needle biopsy. Here, a needle is used to remove a sample. Usually, this is for liquid samples. So, it could be for the lymph, for the blood, and so on. You have a stereotactic biopsy. Here stereotactic system which uses the 3D coordinates is used to identify a small target region and specifically remove tissues. These things are used in places where you need to have a lot more control. For example, if you are going to take brain tissue and so on. So, you would not want to damage the organ. You need to have precise control over which part of the tissue you are harvesting. For that reason, you use something like a stereotactic biopsy.

Student: So, what are the reasons for harvesting brain tissues?

You might want to work with brain cells. There could also be reasons like diagnostics for tumors and so on. So, there are different reasons where you do biopsies.

A colposcopic biopsy is where colposcope is used; this is a close-focused telescope that allows the doctor to look at the cervix in detail. This is usually done for again diagnosing cervical cancer.

Endoscopic biopsy is where an endoscope is used to collect the sample. An endoscope is a long thin, lighted optical instrument, which is used to get deep inside the body, and it can also examine and operate on organs. So, you might have seen endoscopy done through the nose or the mouth; those are very commonly done. So, this is just a long tube that is inserted into your body. These are some of the different techniques which are available readily for performing biopsies.

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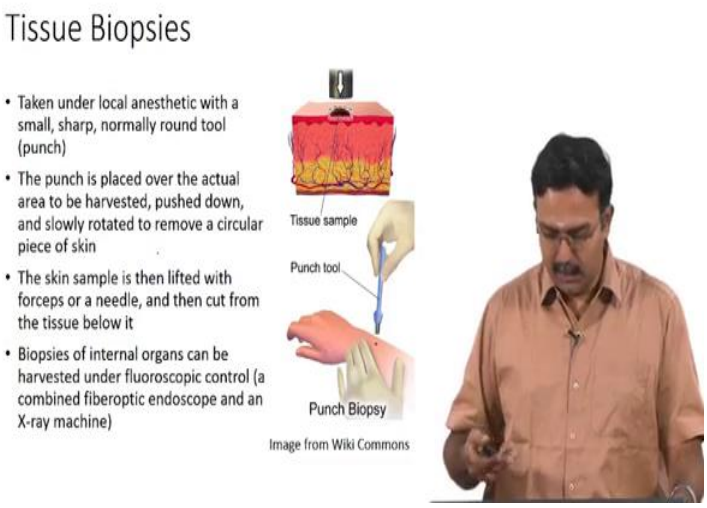

What I am showing here is the punch biopsy. This is one of the simplest things done; usually, this is done for tissues like skin, which are easily cut open. What you do is under local anesthetic, you take this punching tool, which is a sharp knife which is round in shape, and the punch is placed over the area which you want to harvest, and it is pushed down and rotated to remove a circular piece of the skin.

The skin sample is then lifted with the forceps or a needle, and this can be cut from the tissue before it is further processed. Biopsies for internal organs, however, cannot be done in such a simple and crude way. You would need more of a fluoroscopic control; you would either combine it with an endoscope or an X-ray machine so that you actually can keep track of where you are going and what tissue you are removing.

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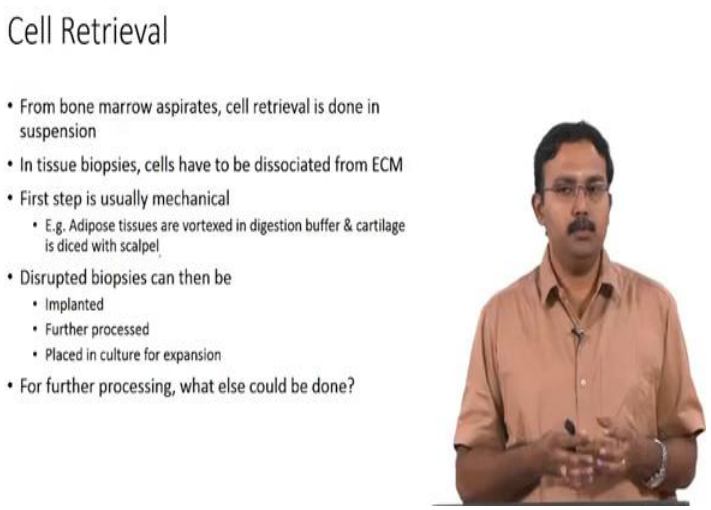

The biopsies you got or the aspirates you got are a mixture of cells. So, you might have to process them further before you can use them for tissue engineering applications. Bone marrow aspirates are suspensions. So, cell retrieval can be done from these suspensions. Whereas tissue biopsies have extracellular matrix attached to it. The cells adhere to the ECM, and you have taken out the tissue. Now, you need to make sure that the ECM is removed before you start working with the cells. If you only want the cells, you do not need the ECM. So, this is the opposite of decellularization. You need to remove the ECM without actually damaging the cells.

The first step is usually mechanical. What you do is, you could either have vortexing with digestion buffer or just dicing with a scalpel and so on. Those are simple techniques to try and remove as much of the ECM as possible. The disrupted biopsies could then be implanted or further processed, or it could be placed in culture for expansion as well. What else do you think you can do for further processing. The first step is just a mechanical right. After that, if you want to remove ECM, what do you think you can do?

Student: Collagenase digestion.

Collagenase or something.

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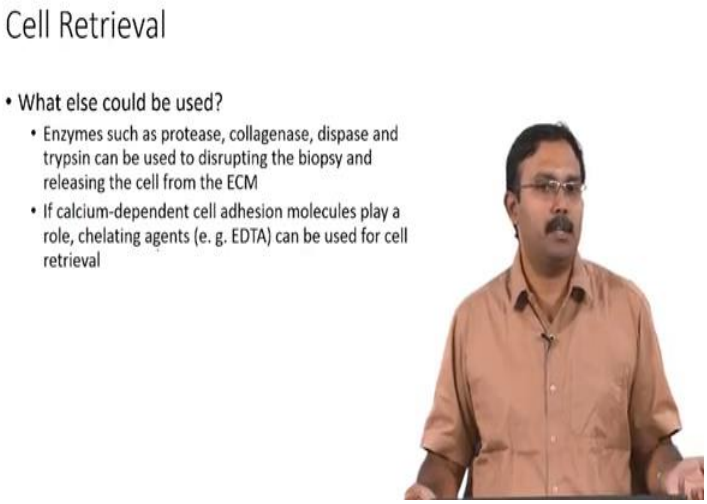

Where you use some protease or enzyme which could disrupt the ECM and release the cells from these ECM, so that would be one way to do this. Trypsin could just dislodge the cells from the ECM. Instead of destroying the ECM, trypsinization makes sure the cells do not adhere to the surface, they just come out of the ECM. So, there are different techniques which you can use.

There can also be calcium-dependent molecules being targeted, which are used for cell adhesion. You can use chelating agents that will target these calcium-dependent cell adhesion molecules and help in the release of cells from the ECM.

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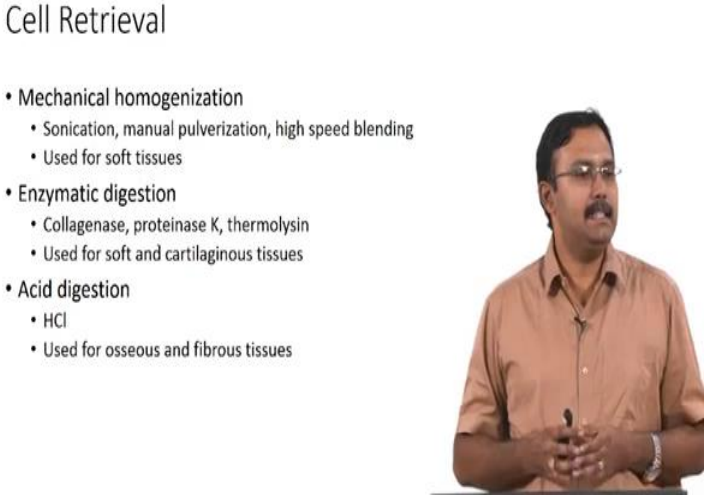

Basically the cell retrieval process can be of two or three different steps. The first step is mechanical homogenization, where you can use sonication, manual pulverization, or high-speed blending; this is usually done for soft tissues. Enzymatic digestion is done for soft and cartilaginous tissues, where you use things like collagenase, proteinase k, and so on. Acid digestion is done for osseous and fibrous tissues. So, instead of using enzymatic digestion when you have hard tissues, you can use acid digestion, which is usually done using HCl.

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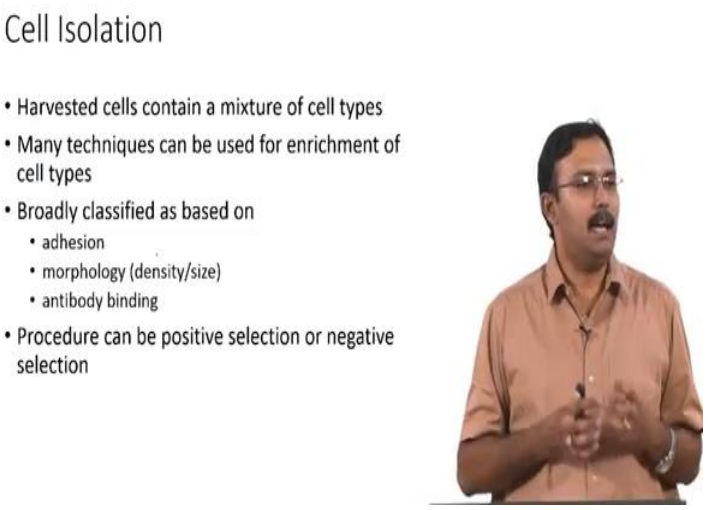

The cells which you have harvested contain a mixture of cell types because you have not done anything to specifically attract one cell type. Whatever you have done till now, you have just done mechanical isolation or enzymatic digestion, which will just retrieve all the cells that are present in the tissue. This would mean there is a mixture of cells. But now you might want to use only one specific cell type. If you want hepatocytes alone, then you would have to make sure that hepatocytes are separated.

There are many techniques that can be used to enrich these cells. So, how you do this is dependent on three major factors. It is based on adhesion properties of the cell, or the morphology of the cell, which would include the density and size of the cell, or it could be antibody binding, so the physiology of the cells and so on. Based on the distinct characteristics which are unique to a cell, you try to separate it from the other cells. So, the procedure can be either a positive selection or a negative selection.

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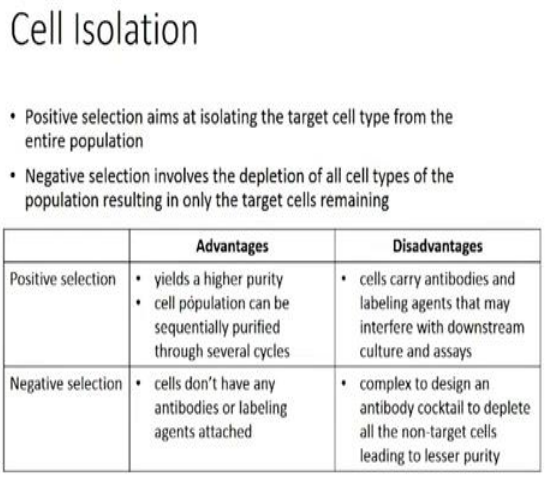

What I mean by that is, in the positive selection, you try to isolate the target cell type from the entire population. You might have like 8 or 10 cell types, and you try to target one cell type and take that out of the mixture. Whereas the other thing is negative selection where you deplete all other cell types, and only the target cells remain, both approaches are possible. Both of them have their own advantages and disadvantages.

In positive cell types, the advantage is there is a high purity because you are targeted one specific cell type; you know for sure only that cell type is going to come. There is going to be a unique property that you identify, and you are going to use that to separate it, so which means it is going to be highly pure. It is not going to have any issues with purity. Also, the cells which you get can be further processed again and again for purification because you have only one type of cell.

And even if a few other things are present with it, if one of the techniques you use is not very specific to one cell type and it can attract two cell types, then what happens is you now again have a mixture, and you can still further process it. But the disadvantage is you have used some antibody or a labeling agent that will attract the cell type of interest. So, your cell might have attached to it, and that could interfere in the further processing. If you are going to culture them or if you are going to use it in vivo, this could be a problem. Yeah, Vasundra.

Student: Sir, Have they tried using same media or something?

So, people do use that. We will talk about that as well; that is also possible, people use selective media. Just like how you do it for transfected cells, but that is primarily done for transfected cells; without transfection, the cells might not have that level of selectivity. In the case of transfection, you can have something like the antibiotic assistance, and then you culture the cells in the presence of the antibiotic, every other cell will die, and only the transfected cell will survive. But if you are talking about cells that are just isolated, it is not very easy to do that.

In negative selection, the problem is it is very complex to design a cocktail that will remove everything except that cell you are interested in. Because you need to now target so many different cell types, so it is a difficult process to do it. Because of this, you will end up with something which is of lesser purity; you may not be able to get the highly pure mixture. However, the advantage is the cells will not have any of the antibodies or labeling agents after you do this.

Both these techniques can be used; you would have to decide on what is of more interest you, what level of purity or whether the cell should not have any antibodies attached, and so on. Depending on what you are going to do with the cells and how you are going to process them, you would choose negative or positive selection techniques.

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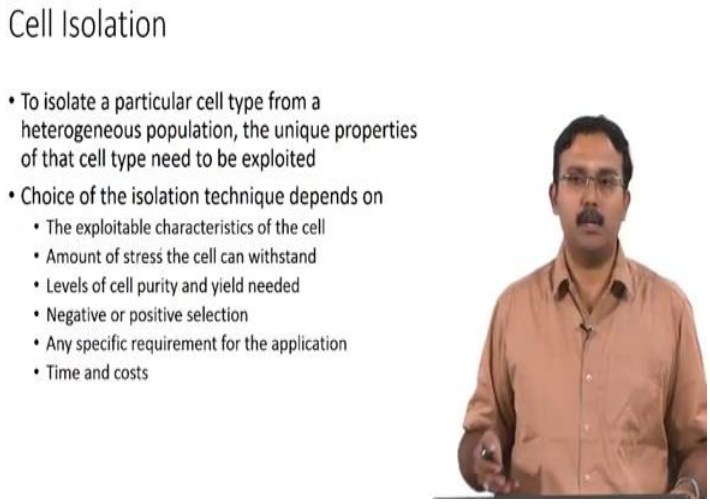

When you are trying to isolate a cell type from a mixture of cells, you want to identify the unique properties of that cell type and try to exploit that. You want to identify

something specific for that cell type. So, the choice of isolation technique will depend on the cell type itself. What do you think are the parameters that will govern the choice of isolation technique? What do you think will affect how the cell isolation is done?

Student: Cell surface marker.

Surface markers are one.

Student: Density.

Cell density, ok.

Student: Size and geometrics.

Size and shape.

Student: Charge.

Cells surface charge ok.

Student: The type of product we finally want.

What do you mean by that?

Student: Depending on the final, I guess.

You mean the application or the product.

Student: Sir application.

So, what you are going to use the cells for ok. Anything else? So

Student: Sir pH resistance.

Ok, pH resistance is one specific aspect. So, it depends on the amount of stress which a cell can withstand. So, you might have cells that can withstand certain pH or certain temperatures or certain sheer, and you can only choose techniques that can handle that. For example, if you are going to use something like a centrifugation technique, cells that cannot withstand sheer cannot be used a separated using centrifugation. You are going to lyse too many cells. So, those are factors which you have to take it account.

Another thing is ultimately what do you want, now how pure of the cells you want, or are you focused on yield if you want isolate as many cells as possible. In some cases, yield is more crucial than purity, you would want to ensure all the cells of the type are recovered, but it is ok if the purity is only 80 percent. So, you might want to use a technique that is suitable for that. Again, negative or positive selection, which will depend on the application we are using.

And any other specific requirement which the application might have. Also, you have to look at time and cost. You cannot have a process that is too time-consuming, which is very low throughput, and it is going to be very expensive, so those things will not be the most attractive option. You need to figure out things that are viable for the specific application you are looking at.

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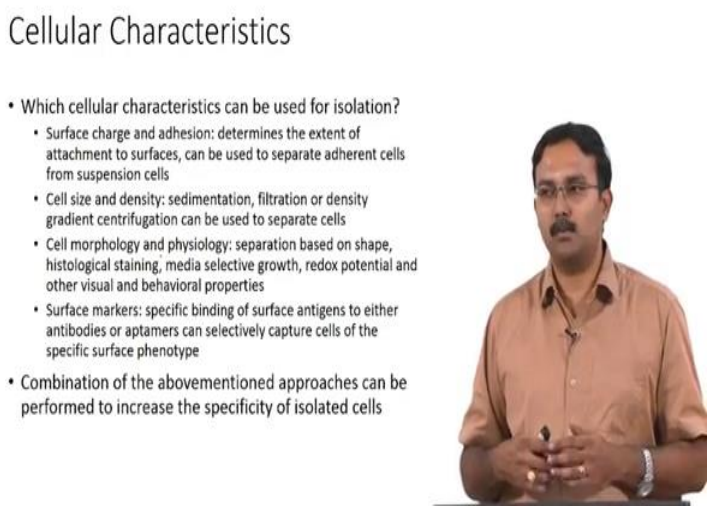

The aspects like surface charge and surface markers would be the cellular characteristics that you are looking to use for isolation. Surface charge and adhesion properties would be important because these determine the extent of attachment to a surface and can be used to separate adherent cells from suspension cells. There are cells that are suspension cells as well; mammalian cells that can survive in suspension are there. These suspension cells can be separated from adherent cells.

Student: So, what are these cells?

Suspension cells.

Student: No, I mean mammalian cells, which can.

Survive under suspense. So, blood cells would be one; all the immune cells are also, they can survive as suspension cells. Cancer cells can survive as suspension cells. Stem cells can also survive as suspension cells. So, mesenchymal stem cells are anchorage-dependent, but there are other adult stem cells and progenitor cells, which are suspension cells. They can form some kind of aggregate and survive as an aggregate. So, we will talk about it.

Cell size and density is a parameter which can be used for separation. So, sedimentation, filtration, density gradient centrifugation are techniques that can be used for separating cells. These have been extensively studied, and people will use it very commonly. Cell morphology and physiology; basically the shape, histological staining, media selective growth, redox potential, and other visual and behavioral properties of the cell, can be used for identifying and isolating the cells. People have used many of these techniques for getting specific cell types.

Last is the surface markers; it is the most specific method. What you do is there is specific binding of a surface antigen either to the antibodies or to aptamers to selectively capture cells that have a specific surface phenotype. You try to attract cells that are of specific interest to you. People also work with a combination of these techniques. Instead of just using one method, people try to use combinations, thereby getting something that is much more pure.

Usually, combinations would include one of the first three features, and the last one because the first three aspects are a lot cruder, compared to the last one right. The last one where you are trying to use surface markers is very specific and also highly sophisticated compared to the other three. Because if you are talking about just surface adhesion, all you are doing is culturing cells.

You are separating adherent cells from non-adherent cells; it is quite simple. And same goes for cell size and density; filtration is a very simple process and so as centrifugation. All you need is, get a suspension of the cells and centrifuge it, and you will be able to get gradient centrifugation done, and you will be able to separate the cells.

Morphology, again those things are not too difficult to do. These are reasonably simple to perform, selective media, and things like that are not too difficult to work with. Whereas, surface markers can actually be quite tricky and quite tedious, because this requires a lot of information about the cell you are interested in, and designing of proper antibodies and antigens, choosing the correct molecules which can actually adhere to the cell you are talking about and using very sophisticated equipment like cell sorters which can actually use this to separate the cells.

So, what people usually do is, perform one of the first three options first and then perform the last one. That way, you do a combination, and you get a much higher chance of higher purity. So, this is very commonly done for getting specific cell types.

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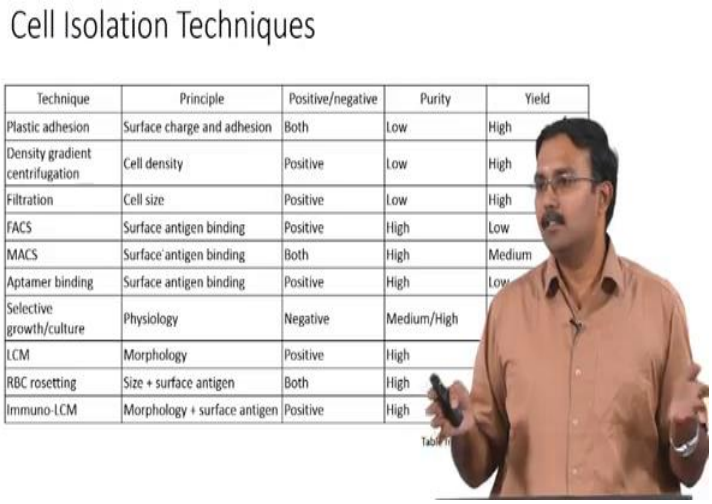

We will go into some of the techniques. This is just an overview of all the techniques that are there. We will talk about individual techniques in greater detail. The first technique is the plastic adhesion, which is based on the surface charge or surface adhesion properties. So, this can either be positive or negative selection. The purity is very low, but the yield is quite high.

All you are doing is segregating; it has two different classes, adherent and non-adherent cells. So, every non-adherent cell is going to be in the suspension; every adherent cell is going to adhere to the surface. So, you are obviously going to get very high yield, but the

purity is going to low because you are going to get a mixture of adherent cells and a mixture of non-adherent cells.

Density gradient centrifugation is based on cell density, and this is a positive selection for separation. And again, the purity which you get would be low because you are centrifugation is not going to make sure it is distinct separations, and again many cells have could similar densities as well. So, you are going to have cell populations in certain regions. The purity is going to be low. And the yield is going to be quite high because, in that particular region, you are going to have all the cells of that particular density.

Filtration is based on cell size. It is a positive selection mechanism where you have low purity and high yield. Again, everything which is larger than the filter pore size is going to be retained, and that is going to be a mixture of cells again. So, you are not able to get one particular cell type from that. FACS, MACS, aptamer binding, or all surface antigen binding techniques which have very high purity and the yield is not very high; yield is low to medium. That is why you try to use the first technique before this so that you make sure you collect all of the cells before you perform further purification. If you have to perform the FACS or MACS or something like that, cells sorter first, then obviously you are not going to have enough to cells to work with.

There can also be a selective growth media or culture where you use the physiology of the cells. Usually, this is negative selection. And here the purity could be medium to high depending on what types of cells you are working with, and what is the mechanism you are trying to use. And yield could again be low to medium. LCM is a laser-based technique that is used for cutting cells, identifying cells, and isolating cells, and that is a morphology-based technique. And this again uses positive selection, and the purity is high, and the yield is low.

With RBC rosetting and immune-LCM, you are trying to use combinations here. RBC rosetting is basically using the size and surface antigen, whereas immune-LCM would be using morphology and surface antigen. These will give you very high purity because obviously you are using the surface antigens as the second technique. So, you are going to have very high purity. Yield will again be medium to low. So, these are the general techniques that have been extensively studied. And we will talk about some of these techniques in greater detail, and some of them we will not discuss.

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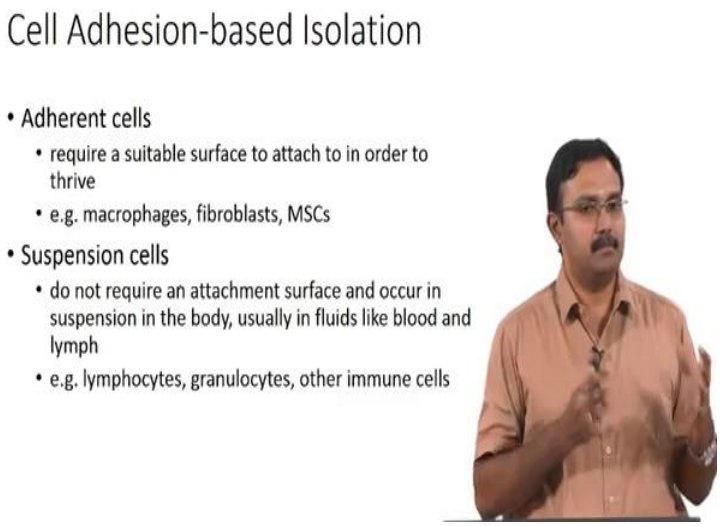

Let us first look at the first criteria, which are adhesion-based isolation. As I said, there are two types of cells, adherent cells, and the suspension cells. Adherent cells require a suitable surface on which they can attach in order to thrive. Examples would be macrophage, fibroblasts, and mesenchymal stem cells. Suspension cells do not require an attachment surface and can occur in suspension in the body. They are usually found in fluids like blood or lymph.

So, all your blood cells are suspension cells. They do not have to adhere to something to grow; they will survive in suspension. Lymphocytes, granulocytes, and other immune cells are all examples of suspension cells. Cancer cells, which have lost the ability to be adherent, are also suspension cells. Some cancer cells, due to the mutations, lose their ability to be adherent. So, they do not have to be adherent. They will be in suspension. So, those cells are also suspension cells.

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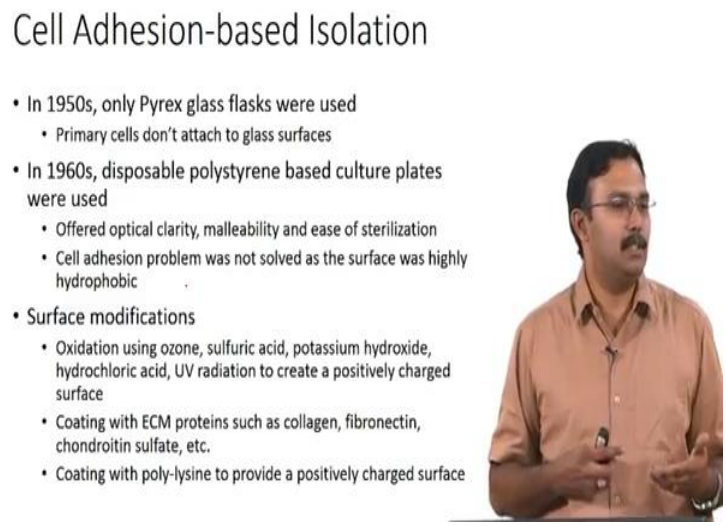

This technique has evolved as the cell culture techniques have evolved. When cell culture was first done, people are using just Pyrex glass flasks on which they are trying to culture the cells. But this does not work because primary cells do not attach to glass surfaces. Are glass surfaces hydrophilic or hydrophobic?

Student: Maybe hydrophobic.

Why do you think it is hydrophobic? Why do you think the glass is hydrophobic? Uncoated glass is hydrophilic; it is not hydrophobic ok. The glasses you see, like what you wear or all the window glasses, all they are coated, so that is why you see droplet standing there. Otherwise, the water spreads very nicely. If you want to perform a contact angle study on a pure glass, the contact angle would be close to 0. So, it is the clean surface that would be very hydrophilic.

But the cells do not like very hydrophilic surfaces as well; they do not like a very hydrophobic or very hydrophilic; they like only something in between. Because of these, the primary cells do not adhere to Pyrex glasses, and also Pyrex glasses were too painful to maintain. You have to sterilize them, and you have to clean them, there is a lot of costs, it will get broken. So, it is very painful to deal with glass culture plates.

In the 60s, disposable polystyrene-based culture plates were brought in, and people starting using them more commonly. They provided the same optical clarity as glass, but they are more malleable and easy to sterilize. So, these were advantages to use them, but the problem was they could not solve the cell adhesion issue because this is highly

hydrophobic. So, your polystyrene is highly hydrophobic. Because of this, cells would not adhere.

Then people started looking at surface modification of these cell culture plates. The first thing was they tried was simple oxidation using ozone or sulfuric acid and potassium hydroxide, hydrochloric acid, and so on. Due to this oxidization, what would have happened is there is a positively charged surface. The cell surface and most of the proteins are negatively charged. Because of these things, cells will adhere. And once they are started adhering, they will start growing, so that is what was tried out as the first surface modification.

Then, people are started coating ECM proteins. Proteins like collagen, fibronectin, chondroitin sulfate have all been coated on this culture plates to make sure that cells will adhere very nicely. And these plates are very effective. Another technique that is being studied is also to use poly-lysine to provide a positively charged surface, again that will help in the negatively charged cells to adhere.

These techniques have improved cell adhesion properties. And now, culture plates are very effective for cell adhesion. Cells will adhere very nicely on culture plates, and they will grow without much of a problem. Now that people have evolved with the cell culture techniques itself, the adhesion based isolation could also be evolved.

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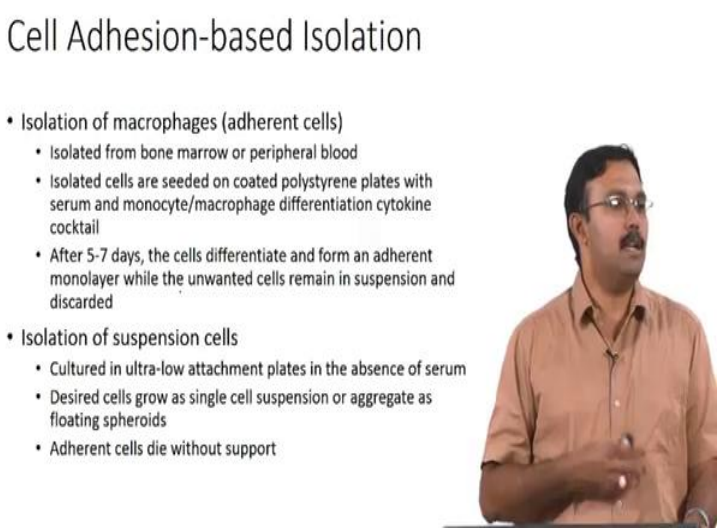

For example, if you want to isolate macrophages which are adherent cells, what you do is you take either bone marrow or peripheral blood, and the mixture of cells are seeded on this polystyrene plate with serum. The monocyte or macrophage differentiation cytokine cocktail is also given, that is the media that is supplied for the cells to grow. After 5 to 7 days, the cells will differentiate and form an adherent monolayer while the unwanted cells remain in the suspension and are discarded.

The stem cells you had in the bone marrow, or peripheral blood would have converted to form macrophages because of the differentiation cytokine cocktail, and then you have only the macrophages which are adhered to the surface. Everything else will be in suspension because the others are all blood cells; they are not going to adhere. This way, you can get macrophages with reasonable purity.

If you are looking at isolating suspension cells, what you do is, you culture it in ultra-low attachment plates in the absence of serum. Now, these plates could either be very hydrophobic or very hydrophilic, and they will not support cell adhesion at all. So, what happens is the cells that can survive single-cell suspensions or aggregates in suspension will survive; other cells will not have any place to adhere, and they will die. So, you will get all the suspension cells only; all the adherent cells would have been killed off.

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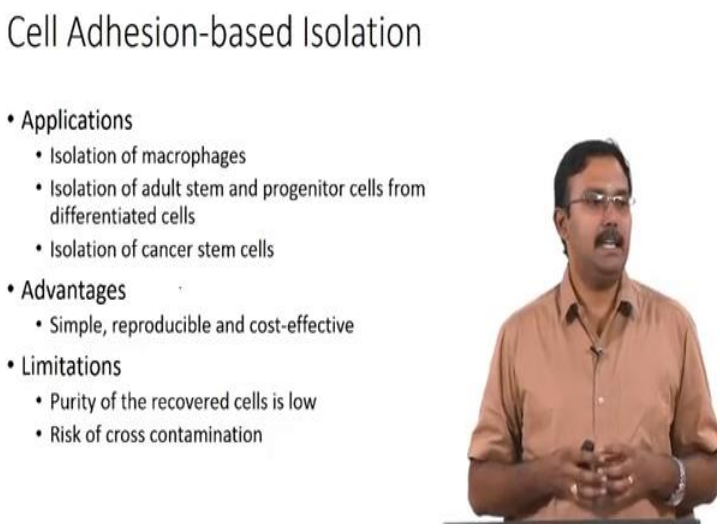

This technique a specifically used for isolation of macrophages and adult stem cells and progenitor cells from differentiated cells and isolation of cancer stem cells. For these

applications, people have used cell adhesion-based isolation. The advantage is it is a simple procedure, which is reproducible and cost-effective, but the limitation is the purity of the recovered cells is low because obviously, you are getting a mixture of cells that could adhere or which could be in suspension and you will get only that. And there is also the risk of cross-contamination. Any time you are performing cell culture, there is a risk of contamination. Contamination is one of the major challenges you are talking about in cell culture.

Because of this, that could also happen, you could always have some bacterial contamination or fungal contamination, which will completely spoil your cultures, so that is a problem. Because of these disadvantages of low purity, people have looked at other techniques that are most specific, if you are looking at getting a specific cell type right. You cannot always purchase cells right; you would have to isolate primary cells from tissues. So, when you have to do that, you have to perform different studies.