METHODS OF CELL CULTURE AND TECHNIQUES USED IN MEDICAL RESEARCH

1. Tissue and cell culture – history and present day.
   1885 - chicken neural plate cultured in a warm saline solution (Wilhelm Roux)
   - 1907 - frog neural tube cultured in frog lymph clot. The formation of nerve processes (Ross Harrison).
   - optimization of culture conditions through the use of different culture media (plasma, serum, extract from chicken embryos) and determination the needs of the cells: osmotic pressure, pH, salts (Burrows, Carrel, Ebeling, Lewis). 20 years of the 20th century - embryonic tissue cultures. The ability of cells to differentiate in culture. Culture in clot on the slide watch.
   40 years of 20th century - the first artificial medium comprising mineral salts, amino acids, vitamins.
   -1952 – the use of trypsin to isolate single cells from tissue and to separate them from the glass (Moscona H, Moscona).
   Modern cell/tissue culture - special devices of laboratory allow to save sterility of culture. Biosafety cabinet - enclosed, ventilated laboratory workspace for safely working with materials contaminated with (or potentially contaminated with) pathogens. All exhaust air is filtered (vertical air flow). CO2 incubator for cell culture maintains optimal temperature, humidity, the carbon dioxide and oxygen content of the atmosphere inside. Plastic disposable dishes, with properly prepared surface, the use of appropriate growth factors for proliferation and growth of differents types of cells, established cell lines.
2. Applications of cell/tissue culture.
   Basic research in vitro, diagnostics, production of vaccines (polio, measles, mumps, rubella), production of enzymes, hormones, cytokines, monoclonal antibodies, getting the cells for transplantation
3. Basic definitions.
   Tissue culture is the growth of tissues or cells in vitro more than 24h (below 24h – incubation.
   Primary cell culture - cell line derived directly from the parent tissue
   Cell line - the cells growing in the first or later subculture from a primary culture (after first passage)
   Cell strain - cells adapted to culture, but with finite division potential
   Established cell line - permanently established cell culture that will proliferate indefinitely given appropriate fresh medium and space (immortal line)
   Diploid cell line - line, wherein at least 75% of the cells have the same karyotype as normal somatic cells of the species from which derived cells
   Heteroploid cell line - cell line, in which less than 75% of the cells has a diploid karyotype.
4. Cell culture media. Osmotic pressure of 340 ± 5 mOsm /kg H2O and the pH in the range 7.2 to 7.4. COMPOSITION OF THE MEDIUM: balanced salt solution, amino acids, glucose, fatty acids, vitamins, purine and pyrimidine bases, phenol red, fetal calf serum – a source of growth factors and hormones.
5. Isolation of single cells or their groups. Culture of the explant (isolated portion of the tissue or organ) for a time sufficient to get out the cells. Separation of explant into

single cells by enzymatic method: a) when small amount extracellular matrix eg. fetal tissues – by trypsin, b) when large amount of extracellular matrix eg. cartilage – by collagenase with deoxyribonuclease. Separation the larger fragments eg. thyroid follicles or pancreatic islets – by collagenase. Separation of a heterogeneous population of cells, eg. blood cells into more homogenous populations a) by density gradient centrifugation. Cells are sorted according to their sedimentation constant, b) by passing through a magnetic column with beads conjugated with antibodies. Cells are sorted depending on their surface antigens (magnetic assisted cell sorting).

6. Contact inhibition. mechanism which functions to keep cells growing into a layer one cell thick (a monolayer). If a cell has plenty of free space, it divides rapidly. This process continues until there is no any room in the layer for cells. At this point, normal cells will stop dividing. Contactinhibin – membrane glycoprotein from human diploid fibroblasts, causes a reversible inhibition of proliferation in confluent culture. CiR – plasma membrane protein - a receptor for contactinhibin mediating the contact-dependent inhibition of growth of cultured cells. The ligand-receptor binding causes changes in the cyclin-dependent kinases (inactive Cdk4), which leads to inhibition of cell division. This effect is seen also with other cell types, with the exception of transformed cells, which do not show the phenomenon of contact inhibition. Transformed fibroblasts – lack of CiR.
7. Hayflick limit - normal human fibroblasts derived from young person can divide – several dozen x (40 to 60) = several dozen population doublings (shortening of telomeres, replicative senescence). Linear chromosomes are less stable than circular, but determine the genetic diversity of living organisms (recombinations). However, the 3 'and 5' ends are sensitive to DNA-degrading enzymes, as well as linear chromosomes may undergo fusion. Telomeres - repetitive non-coding DNA sequences (TTAGGG) with proteins, located at the ends of chromosomes, prevent the damage of chromosomes. During each replication cell loses 50-200 bp telomeric DNA. When the telomeres reach a critical length - STAGE OF CELLULAR SENESCENCE (p53-dependent inhibition of cell cycle).
8. Microscopy: one of the earliest tools of the cell biologists. The first compound microscope appearing in 17th century in Netherlands (inventor Cornelis Drebbel, Hans Lippershey, Zacharias Janssen). Antonie van Leeuwenhoek discovered red blood cells and spermatozoa and helped popularise microscopy as a technique. On 9 October 1676, Van Leeuwenhoek reported the discovery of microorganisms.
9. CYTO- AND HISTOCHEMISTRY - detection of chemical compounds present in the cells, tissues, and in the matrix. Histochemical color reaction, autoradiography, immunohistochemistry.
10. Histochemical color reaction. Reaction P.A.S. - Periodic Acid- Schiff stain- detection of polysaccharides. Polysaccharides, periodic acid, (oxidation of the glycol groups – vicinal diols), aldehyde groups, Schiff reagent (colorless basic fuchsin), red color of Schiff reagent. (GLYCOGEN STORAGE DISEASES, CANCERS)
11. Histoenzymology. Enzyme, (eg. alkaline phosphatase, succinic acid dehydrogenase), substrate, product, intermediate compound, colorful precipitate (insoluble). Example: detection of alkaline phosphatase: substrat - beta-naphthyl phosphate, alkaline phosphatase dephosphorylates substrat and beta-naphthol is formed. Diazonium dye is

converted into color complex. (TUMORS DERIVED FROM THE TROPHOBLAST, METASTASIS OF PROSTATE CANCER)

12. Autoradiography. Incubation tissue with isotope. Isotope will emit radiation (usually beta). Exposition to film or emulsion (AgBr in gelatin). Radiation will hit silver grains in emulsion and expose them. Formation of latent image; metallic silver. Photographic processing transforms the latent image into a visible image and makes this permanent. Over the place where was isotope incorporated are visible black grains of silver.
13. Immunohistochemistry. Detection of the antigens present in cells and tissues using antibodies: monoclonal or polyclonal. Methods for labeling antibodies: fluorochromes (eg. fluorescein derivative), metals (eg. ferritin, colloidal gold), isotopes (eg. ^{3}H, ^{125}I, ^{35}P, ^{14}C), enzymes (eg. alkaline phosphatase, peroxidase). Direct method - with using primary antibodies, indirect method with using primary and secondary antibodies. Biotynylated secondary antibody, biotynylated marker, streptavidin or avidin.
14. Flow cytometry. A flow cytometer has a measuring system, optical systems – lamps, lasers resulting in light signals, a detector and Analogue-to-Digital Conversion (ADC) system - which generates forward-scattered light (FSC) and side-scattered light (SSC) as well as fluorescence signals from light into electrical signals that can be processed by a computer. The cells pass single through the light beam and cause FSC and SSC of laser beam. FSC – Forward-scattered light is proportional to cell-surface area or size -the greater cell the greater deflection or FSC. SSC - Side-scattered light is proportional to cell granularity or internal complexity. The more complicated cell the greater dispersion of light or SSC. When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the surface markers of the cell. Different fluorochromes can be used to distinguish subpopulations. The staining pattern of each subpopulation, combined with FSC and SSC data, can be used to identify cells. Cytometer first needs to identify the cells of interest, then separate out the individual cells. Sorting allows us to capture and collect cells of interest for further analysis. Once collected, the cells can be analyzed microscopically, biochemically, or functionally. FLOW CYTOMETRY APPLICATIONS: DIAGNOSIS: proliferative diseases of the hematopoietic and lymphatic system, congenital and acquired immunodeficiency, autoimmune diseases, analysis of cells for bone marrow transplantation, MONITORING: the treatment of leukemia, the immune system of HIV patients, immunosuppressive treatment of transplanted patients.
15. Material isolation: ultrasonic cell disruption, the breakdown of cell membranes by detergents, mechanical disruption. Fractionation: differential centrifugation based on differences in size and density, with larger and denser particles pelleting at lower centrifugal forces.
16. Western blot. Method used to detect specific proteins in a sample of tissue homogenate, cellular extract or in body fluid. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Proteins in the sample are heated with the negatively charged detergent SDS (anionic surfactant), which unfolds them and coats them with a uniform negative charge density; disulfide bonds (S-S) are reduced with mercaptoethanol. The sample is applied to the well of polyacrylamide gel, and a voltage is applied to the gel. The negatively charged

detergent-protein complexes migrate to the bottom of the gel, toward the positively charged anode. Small proteins move faster, larger proteins move slower, so proteins are separated by size, smaller toward the bottom and larger toward the top. 2. Transfer proteins from gel to membrane. The gel with the resolved set of proteins is then placed in an apparatus that permits electrophoretic transfer of the proteins from the gel onto the surface of a special paper (e.g., nitrocellulose paper, polyvinylidene fluoride (PVDF) membrane) to which proteins strongly adsorb. 3. Methods of detection. Immunohistochemistry .Colorimetric, chemiluminescence, fluorescence.

17. ELISA assay. Detection and quantification of specific protein using antibodies conjugated with enzymes. 96-well plate with specific capture antibody coating. The target protein-containing samples and serial dilutions of standard with known concentration are applied to the plate, and molecules of target protein are captured by antibodies. Enzyme-linked antibodies are applied as detection antibodies that also bind specifically to target protein. Substrate addition. Conversion of colorless substrate into a colored product. The intensity of color depends on amount of target protein. The absorbance of the plate wells is measured to determine the presence and quantity of target protein. . The absorbance (optical density) of each well is read with a plate reader (kind of spectrophotometer). Standard curve is prepared from the data produced from the serial dilutions of standard with concentration on the x axis (log scale) vs absorbance on the Y axis (linear). The concentration of the sample is interpolated from this standard curve.
18. Properties of DNA. Denaturation - or DNA melting is the separation of a double strand into two single strands, which occurs when the hydrogen bonds between the strands are broken. (thermal denaturation – by heat, chemical denaturation – by bases, acids). Renaturation - formation of double stranded DNA from denatured complementary DNA strands caused by slow cooling. Hydrogen bonds are formed. Hybridization - a phenomenon in which single-stranded DNA or RNA molecules spontaneously anneal to complementary DNA or RNA.
19. Southern blot is a method used in molecular biology for detection of a specific DNA sequence in DNA samples. The method is named after its inventor, the British biologist Edwin Southern. 1.Restriction endonucleases are used to cut DNA into small fragments. 2.The electrophoresis of DNA fragments on an agarose gel to separate them by size. 3. Alkaline DNA denaturation. 4. Transfer DNA from gel onto nitrocellulose or nylon membrane. 5. The membrane is exposed to a hybridization probe - a DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected. Southwestern blot is used for identifying and characterizing DNA-binding proteins. The proteins are separated by electrophoresis, transferred to membrane and detected by DNA probes.
20. Northern blot - technique used to study gene expression by detection of RNA in a sample. It was used for determining the particular gene expression. Extraction of total RNA from cells. RNA samples are separated by gel electrophoresis and transferred to a nylon membrane. Complementary labeled probe is hybridized to the RNA on the membrane.
21. Hybridization *in situ*. Breast cancer - HER2 gene multiplication (probe HER2/CEN-17. (Green signal with HER2 and orange of the centromere of chromosome 17).

22. Polymerase Chain Reaction. Thermus aquaticus is a species of bacterium that can tolerate high temperatures. It is the source of the heat-resistant enzyme Taq DNA polymerase, most important enzyme in molecular biology because of its use in PCR reaction - DNA amplification technique. It was first discovered in the Lower Geyser Basin of Yellowstone National Park. Heat-stable DNA polymerase - Taq polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. Detection of specific mRNA: 1. Isolation of mRNA from cells. 2. Reverse transcription of mRNA to complementary DNA. PCR reaction. Agarose gel electrophoresis (DNA staining with ethidium bromide).
23. Real time PCR - quantification of gene expression. Measurement after each cycle. Mathematical quantification. Labeled probes or primers (appropriate DNA sequence). Fluorescent report dye (R), and quencher (Q) are attached to the 5' and 3" ends of probe. When the probe is intact the R emission is quenched. During each cycle the DNA polymerase cleaves the report dye from the probe. Once separated from the quencher, the report dye emits characteristic fluorescence. This fluorescence is measured and analyzed by special software for analyzing the data.

PRACTICAL PART.

Slides

1. Smear of lymphocytes – slide 204

2. Smear of granulocytes - slide 205 – recognize the population of granulocytes

3. Cultured human diploid fibroblasts (slide 97a) - confluent culture - contact inhibition

4. Cultured heteroploidal human fibroblast transformed with cowpox virus (slide 300) - pay attention to the different shape of fibroblasts and the lack of contact inhibition

5. Reaction PAS in the liver cells (slide 222a) – in the cytoplasm of cells granules of glycogen are visible. Because they are displaced during the preparation of the slide they are often located at one pole of the cell.

6. Reaction PAS in the small intestine (slide 222 b) - goblet cells, which produce mucus are stained red color. Nuclei are stained with hematoxylin.

7. Reaction with alpha-naphthyl phosphate and the diazonium dye for detection of alkaline phosphatase activity in the kidney (slide 220a). Alkaline phosphatase is present in the brush border of proximal tubules.