Introduction and Disclaimer

These mock examination questions span diverse disciplines and are designed for your practice in preparation for the International Research Olympiad (IRO) 2024. Endeavor to answer them to the best of your ability, utilizing this opportunity to enhance your skills and knowledge. For additional practice, it is advisable to engage in extensive reading of various papers; such efforts will contribute to a more comprehensive and nuanced understanding of the subject matter.

Try your best, and good luck! -International Research Olympiad 2024

A non-aggressive, highly efficient, enzymatic method for dissociation of human brain-tumors and brain-tissues to viable single-cells

Abstract:

Background: Conducting research on the molecular biology, immunology, and physiology of brain tumors (BTs) and primary brain tissues requires the use of viably dissociated single cells. Inadequate methods for tissue dissociation generate considerable loss in the quantity of single cells produced and in the produced cells' viability. Improper dissociation may also demote the quality of data attained in functional and molecular assays due to the presence of large quantities cellular debris containing immune-activatory danger associated molecular patterns, and due to the increased quantities of degraded proteins and RNA.

Results: Over 40 resected BTs and non-tumorous brain tissue samples were dissociated into single cells by mechanical dissociation or by mechanical and enzymatic dissociation. The quality of dissociation was compared for all frequently used dissociation enzymes (collagenase, DNase, hyaluronidase, papain, dispase) and for neutral protease (NP) from Clostridium histolyticum. Single-cell-dissociated cell mixtures were evaluated for cellular viability and for the cell-mixture dissociation quality. Dissociation quality was graded by the quantity of subcellular debris, non-dissociated cell clumps, and DNA released from dead cells. Of all enzymes or enzyme combinations examined, NP (an enzyme previously not evaluated on brain tissues) produced dissociated cell mixtures with the highest mean cellular viability: 93 % in gliomas, 85 % in brain metastases, and 89 % in non-tumorous brain tissue. NP also produced cell mixtures with significantly less cellular debris than other enzymes tested. Dissociation using NP was non-aggressive over time—no changes in cell viability or dissociation quality were found when comparing 2-h dissociation at 37 °C to overnight dissociation at ambient temperature.

Conclusions: The use of NP allows for the most effective dissociation of viable single cells from human BTs or brain tissue. Its non-aggressive dissociative capacity may enable ambient-temperature shipping of tumor pieces in multicenter clinical trials, meanwhile being dissociated. As clinical grade NP is commercially available it can be easily integrated into cell-therapy clinical trials in neuro-oncology. The high quality viable cells produced may enable investigators to conduct more consistent research by avoiding the experimental artifacts associated with the presence dead cells or cellular debris.

Key Terms:

- **DAMPs (Danger-Associated Molecular Patterns)**: Molecules released by stressed or damaged cells that can activate the immune system
- **GMP (Good Manufacturing Practice):** Set of regulations and guidelines for the manufacturing of pharmaceuticals and other products
- **Forward Scatter (FS):** Measurement of light scattered in the forward direction as cells pass through a laser beam
- **Viability Dye Channel:** Used to distinguish live cells from dead cells
- **Multicolor Flow Cytometry (FCM):** Flow cytometry analysis using multiple fluorescent markers, allowing for simultaneous detection of multiple parameters
- **Immunotherapy Trial:** Using the body's immune system to treat diseases, including cancer
- **Cell Sorting Experiments:** separating different types of cells based on a specific characteristic
- **Ambient Temperature Shipping:** Shipping or transporting materials at room temperature without the need for special temperature controls

Paper:

Investigating the physiology, molecular biology and immunology of brain BTs frequently requires the use of viable single cells produced by dissociation of tumor pieces collected from patients undergoing craniotomy. Several methods are used to dissociate the tumor mass into viable single cells. These include mechanical dissociation (e.g. meshing, trituration with a pipette/tip)], enzymatic digestion, or a combination of both. Enzymes such as papain, dispase, collagenase, hyaluronidase, DNase, and trypsin are commonly used for dissociation, either alone or in combination. Enzymes dissociate the cell–cell contacts and the extracellular matrix (ECM) encompassing cells within the brain tissue or inside the BT.

The various dissociation methods largely differ in their yield of cells and in the percentage of viable cells produced. The produced cell mixtures (i.e. the cells and their surrounding solution) may differ in their dissociation quality i.e. the undissociated cell clumps, the extent of subcellular debris, and the amount of spilt nucleic acids.

Inefficient or overly aggressive tumor dissociation may cause the release of cellular materials that constitute DAMPs or alarmins. Such materials include glutamate, ATP, HMGB1, and others. The released cellular components may activate, modulate or selectively kill the assayed cells thereby producing significant experimental artifacts. Inappropriate tissue dissociation may also compromise the quality of functional assays that require intact viable cells. It may reduce the accuracy of the results of molecular assays such as gene expression assays that

require genetic material of suitable integrity, and may alter the results of flow cytometry (FCM) that correctly analyze only intact single cells.

In addition to their use in research, brain tumor cells dissociated from surgical specimen are used in clinical trials for production of whole-cell vaccines. Vaccination with live, dead or dying cells results in different immunological responses. In preparation for a clinical trial using viable dissociated glioblastoma cells as vaccines, we sought an optimal dissociation method that could produce single cells of the highest possible viability and of the optimal dissociation quality using enzymes approved for clinical use.

To evaluate which enzyme or enzyme combination produces single cells of the highest dissociation quality from dissociated brain lesions, all commonly used enzymes were tested on a large set of non-tumorous brain lesions and BT samples. Our results show that NP from Clostridium histolyticum, an enzyme not previously used on human brain lesions, produced single cells of the highest viability and cell mixtures of the finest dissociation quality. NP's non-aggressive nature enabled long term incubations with no apparent reduction in the dissociated cells' viability or in the dissociation quality.

Here we investigated all widely used methods and enzymes employed for dissociation of BTs and brain tissue using the largest panel of tissue samples used for such comparison. Unlike previous work, we added a visual grading system, CG, for the evaluation of the dissociation quality component of the produced cell mixtures.

Cell mixtures of lower dissociation quality generally yielded fewer cells. More importantly, cell mixtures of higher dissociation quality contained less components released from dead or dying cells. Cellular debris contains DAMPs, substances to which brain-resident immune cells, and brain-infiltrating immune cells respond. The presence of DAMPs in large quantity may alter the results of functional experiments using the dissociated cells.

While DCH, the most widely used method to produce single cells from human BTs, generated single cells of similar viability as that of dispase, it produced mixtures of significantly lower dissociation quality. Although dispase produced cell mixtures of acceptable viability and dissociation quality, there is no commercially available clinical-grade version of this enzyme. In contrast, NP is an inexpensive enzyme which is available in clinical and non-clinical grades. Importantly, NP was found to dissociate brain tissues significantly better than dispase, both in regard to cellular viability and to dissociation quality.

In addition to NP's ability to gently dissociate brain/ tumor tissue for short duration (2 h), it dissociated tissues for longer durations at ambient temperature without any apparent reduction in the produced cellular viability or the dissociation quality.

Neutral proteases are not inhibited by serum and can be used in cell culture media to inhibit formation of cell clumps. Thus it may be possible to transport brain tissues or BTs at ambient temperatures in tissue-culture medium with or without serum, supplemented with NP. The tissue obtained from patients at one clinical site could be sent in culture medium with NP,

and processed as fresh tissue at a distant site. This may facilitate multicenter collaborations requiring centralized processing of fresh tissue samples.

NP is not of eukaryotic origin, thus carries no risk of spongiform encephalopathy. Its clinical-grade version is made under GMP guidelines, and was previously used in trials in which the dissociated cells, e.g. pancreatic islet cells were returned to humans. This enables the simple integration of this enzyme into clinical trials in the field of neuroscience.

Figure 5d demonstrated some discrepancy between cellular viabilities evaluated by trypan-blue and by FCM. This previously reported discrepancy is likely due to the fact that microscopy and FCM identify differently what is "a cell". Microscopy identifies cells via their shape; while blood cells are microscopically easily identifiable, brain or BT cells are highly irregular (see Fig. 5b, c) and sometimes difficult to identify. FCM, on the other hand, identifies cells by their light scatter characteristics. "Cells" are electronically collected "events" above a somewhat arbitrary forward scatter threshold. Also in FCM, cellular identification is complicated by the irregularity of the cells and the high variability in their sizes. Another complicating factor for FCM is that the dissociated cell mixtures may contain large amounts of cellular debris. While the use of an amine dye does a good job at discriminating between live and dead cells, it is less efficient in discriminating between live cells and debris, both having low fluorescence in the viability dye channel.

Which method should be used to evaluate viability? Microscopy may be better at correctly identifying cells and more widely accepted by regulatory agencies. On the other hand FCM is rapid, quantitative and more userindependent thus enabling standardization of analysis and comparison of viabilities across different samples dissociated by different labs.

Using the high dissociation quality cell mixtures produced from BTs and brain samples enables our lab to run elaborate multicolor (up to 10 colors) FCM analyses and FCM sorting experiments of intratumoral cells. When using the dissociated BT cells in functional immune assays (e.g. co-culturing of tumor cells with lymphocytes) we see that cell mixtures of low dissociation quality yield atypical results.

Calibration of an optimal way to dissociate brain tissues or BTs into viable cells is important both clinically and scientifically. Clinically, intact BT cells used for immunotherapy trial should contain minimal amounts of debris, and maximal amounts of viable cells, whether cells are viable cells or irradiated. Scientifically, the production of better quality cell mixtures is the first important step for attaining more consistent and reliable results in the field of neuroscience.

Neutral protease (NP) from Clostridium histolyticum, an enzyme previously not used in the field of neuroscience, dissociates human brain tissue and brain tumors to single cells with significantly higher viabilities and cleaner cell-mixtures than all other widely-used enzymes. The non-aggressive nature of NP allows for tissue dissociation for extended durations, enabling for ambient-temperature shipping of fresh tissue pieces meanwhile being dissociated.

Improper tissue dissociation may reduce the quality of data attained in functional and molecular assays due to the presence of large numbers of necrotic cells, spilt nucleic acids, and

the presence of subcellular debris, containing immune-activatory danger associated molecular patterns (DAMPs). Production of high-quality viable single cells from brain tissue is the first step for more consistent and reliable results in the field.

Paper 7: Neurology

Question 1

Question: Regarding the motivations behind the paper, which of the following examples are noted to specifically demonstrate the importance of high cell viability?

a.) The inadequate release of molecules regulating immune functions.

b.) Poorly dissociated cell mixtures with clumps and debris.

c.) Assays that require feasible single cells.

d.) Clinical applications for vaccinations.

Question 2

Question: What was the primary reason for testing different enzymes, including neutral protease (NP), in the dissociation of brain tumor tissues?

a.) To find the enzyme that is least expensive.

b.) To identify the enzyme that produces the highest mean cellular viability.

c.) To compare the speed of dissociation among different enzymes.

d.) To analyze the effects of enzymes on protein structures in cells.

Question 3

Question: According to the paper, which of the following is the best way to describe how the tissue samples are prepared in the step preceding enzymatic testing?

a.) Removing interfering tissues and blood clots.

b.) Using mechanical dissociation techniques.

c.) Staining with trypan blue dye to distinguish between alive and dead cells.

d.) Mixing the portioned samples with a plastic Pasteur pipette.

Question 4

Question: How does the stability of cell viability and dissociation quality over extended periods of time at varying temperatures, as observed with NP, impact the potential for its use in multi-center clinical trials and neuro-oncology research?

a.) It suggests that NP can only be used in single-center trials.

b.) It indicates that NP is not suitable for long-term cell viability studies.

c.) It implies that NP-dissociated tissues can be transported at ambient temperature, facilitating multi-center trials.

d.) It shows that NP is effective only at a specific temperature, limiting its use in varied clinical settings.