

Non-invasive imaging of hypoxia in tissue engineering

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Introduction:

In tissue engineering, cells are grown on biomaterials in vitro and subsequently implanted. A critical parameter in effective proliferation and differentiation is the availability of nutrients. Few tools are currently available to monitor the nutritional status of cells. In this study, we have employed A4-4 cells [1], a Chinese hamster ovary cell line stably transfected with a luciferase gene driven by the hypoxia responsive element (HRE) from the promoter region of the VEGF gene [2, 3]. HRE activity, and thus luciferase activity, directly correlates with decreasing cellular O₂ levels.

The aim of this study is to investigate whether the HRE-luciferase construct can be used for non-invasive imaging of hypoxia in tissue engineering.

Materials and Methods:

A4-4 cells, kindly provided by dr. Nose, were cultured in 96-well plates with different initial cell numbers per well. After 24h attachment in normoxia, cells were exposed to hypoxia (5% CO₂ and 95% N₂) or normoxia (5% CO₂ and 95% air) for 8h. Luciferase activity and cell numbers were determined by Luciferase Assay System and CellQuant Assay respectively. For 3D culturing, cells were seeded on ceramic biphasic calcium phosphate particles, and cultured for 1 day and 7 days. Furthermore, A4-4 cells were seeded in agarose and the viability was analyzed by ethidium homodimer /calcein staining. Subsequently, luciferase activity was measured by bioluminescent imaging using a CCD camera by incubating the gels in a luciferin solution and imaging at 1 minute intervals for 1 hour. The gel was 1% (w/w) and the size was 4.8 x 4.8mm.

Results:

The effect of various cell densities on hypoxia in their environments is shown in Fig.1. In normoxia, a cell density dependent increase in luciferase activity was observed at 4x10⁴ cells/gel and higher. In contrast, hypoxia treatment for 8h induced expression even at lower cell densities, demonstrating that the cells respond to hypoxia as reported before [1].

Next, we seeded the cells on BCP particles. On day 1, it was very clear that higher cell number caused higher hypoxia in their environment. But on d7 the difference was not obvious, and the cellular luciferase on d 7 was even lower than d 1. We are currently repeating this experiment.

24 hours after seeding cells in agarose gels, most cells were alive (Fig.3a,b), but massive cell death occurred after 2 days (data not shown). Luciferase activity was measured 24 hours after seeding.

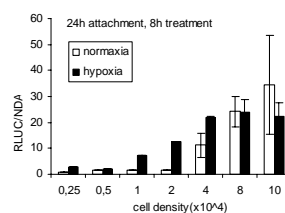


Fig.1 Relative cellular luciferase activity of 2D culturing in normoxia and hypoxia.

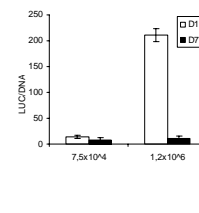


Fig.2 Cellular luciferase activity of 3D culturing on BCP.

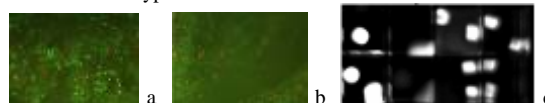


Fig.3 Fluorescence images of a section of agarose gel by viability staining for 1x10⁶ cells (a) and 2.5x10⁶ cells (b) per gel after 1d. (c). Bioluminescence image of the gels after 1d.

Fig.4a showed that the light intensity increased with time after addition of the luciferin substrate to the gels and then reached a plateau, demonstrating the diffusion of the luciferin substrate into the gel. The plateau of light intensity from 2.5million cells samples is 1.15 fold higher than that from 1 million cells samples (Fig.4b).

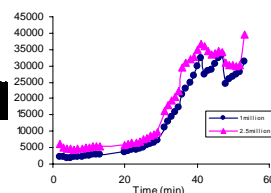


Fig.4a. Luciferase activity at various time points after luciferin administration (Diamond: 1x10⁶ cells/gel; triangle: 2.5x10⁶ cells/gel).

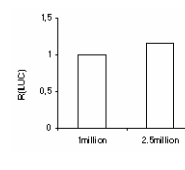


Fig. 4b. Plateau level of luciferase activity.

Discussion:

In this study we demonstrated that the HRE-luciferase construct can be used to report hypoxia, both in cells on culture plastic and on biomaterials. The relative slow diffusion of the luciferin substrate into 3D scaffolds complicates the analysis but also provides information about the properties of the respective materials. Future research will focus on hypoxia during in vitro expansion of cells and upon implantation in animal models.

References

- [1] Yohko Yamazaki, *et al Biol. Pharm. Bull.* **26**(4) 417-420 (2003); [2] J. Mogford, *et. al, Wound Repair and Regeneration.* **11**(6) 496-503 (2003); [3] Toru Shibata, *et. al, Int. J. Radiation Oncology Biol. Phys.,* **42**(4) 913-916 (1998)