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在线全文

不同刚度Ⅰ型胶原凝胶三维环境调控NK细胞免疫功能*

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【摘要】 **目的** 构建不同刚度Ⅰ型胶原凝胶并探讨其三维(three-dimensional, 3D)培养环境对自然杀伤 (natural killer, NK) 细胞形态、自由迁移能力以及细胞杀伤功能的影响。**方法** 分离Sprague Dawley(SD)大鼠鼠尾Ⅰ型胶原蛋白并制备不同刚度的胶原凝胶，通过激光共聚焦显微镜观察其微结构，流变仪测量平台期的储能模量表征其刚度。将NK-92MI细胞培养于不同刚度Ⅰ型胶原凝胶中，倒置显微镜观察NK-92MI细胞的形态，高内涵成像系统记录NK-92MI细胞的自由迁移过程，分析迁移速度和距离。不同刚度的Ⅰ型胶原凝胶培养NK-92MI细胞24、48 h，再与人结直肠腺癌上皮细胞（DLD-1）共培养后，用CCK8法检测DLD-1细胞增殖率，分析NK-92MI细胞的细胞杀伤能力。**结果** 成功制备出刚度为（10.97±2.10）Pa的低刚度Ⅰ型胶原凝胶和刚度为（114.50±3.40）Pa的高刚度Ⅰ型胶原凝胶。与在低刚度Ⅰ型胶原凝胶中相比，在高刚度Ⅰ型胶原凝胶中，NK-92MI细胞呈现更细长的形态（$P<0.05$），细胞平均面积减少〔（69.88±26.97）μm^2 vs.（46.59±21.62）μm^2，$P<0.05$〕，细胞圆度减小（0.82±0.12 vs. 0.78±0.18，$P<0.05$），细胞迁移速度降低〔（2.50±0.91）μm/min vs.（1.70±0.72）μm/min，$P<0.001$〕，迁移距离缩短〔（147.10±53.74）μm vs.（98.03±40.95）μm，$P<0.0001$〕，差异均有统计学意义。与低刚度Ⅰ型胶原凝胶相比，高刚度Ⅰ型胶原凝胶培养24 h的NK-92MI细胞可促进DLD-1细胞增殖〔增殖率（46.39±12.79）% vs.（65.87±4.45）%，$P<0.05$〕，降低细胞杀伤能力，48 h的比较结果类似〔增殖率（31.36±2.88）% vs.（74.57±2.16）%，$P<0.05$〕，差异均有统计学意义。**结论** 不同刚度Ⅰ型胶原凝胶3D培养环境可改变NK-92MI细胞的形态、迁移能力以及杀伤功能。该研究为探索和理解生物力学微环境影响NK细胞免疫应答机制提供了研究基础，为优化免疫治疗方案提供理论依据。

【关键词】 Ⅰ型胶原 细胞外基质 力学 杀伤细胞 免疫应答

The Three-dimensional Environment of Type Ⅰ Collagen Gels With Varying Stiffness Modulates the Immunological Functions of NK Cells

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【**Abstract**】 **Objective** To construct type Ⅰ collagen gels with different stiffness and to investigate the effects of three-dimensional (3D) culture environments of the gels on the morphology, free migration ability, and cell killing function of natural killer (NK) cells. **Methods** Type Ⅰ collagen was isolated from the tails of Sprague Dawley (SD) rats and collagen gels with different levels of stiffnesses were prepared accordingly. The microstructure of the collagen gels was observed by laser confocal microscopy. The stiffness of the collagen gels was assessed by measuring the plateau modulus with a rheometer. NK-92MI cells were cultured in collagen gels with different levels of stiffness. The morphology of NK-92MI cells was observed by inverted microscope. High content imaging system was used to record the free migration process of NK-92MI cells and analyze the migration speed and distance. NK-92MI cells were cultured with type Ⅰ collagen gels with different levels of stiffness for 24 h and 48 h and, then, co-cultured with human colorectal DLD-1, a human adenocarcinoma epithelial cell line. CCK8 assay was performed to determine the proliferation rate of DLD-1 cells and analyze the cell killing ability of NK-92MI cells. **Results** Low-stiffness type Ⅰ collagen gel and high-stiffness type Ⅰ collagen gel with the respective stiffness of (10.970±2.10) Pa and (114.50±3.40) Pa were successfully prepared. Compared with those cultured with the low-stiffness type Ⅰ collagen gel, the NK-92MI cells in the high-stiffness type Ⅰ collagen gel showed a more elongated shape ($P<0.05$), the mean area of the cells was reduced ([69.88±26.97] μm^2 vs. [46.59±21.62] μm^2, $P<0.05$), the roundness of the cells decreased (0.82±0.12 vs. 0.78±0.18, $P<0.05$), cell migration speed decreased ([2.50±0.91] μm/min vs. [1.70±0.72] μm/min, $P<0.001$) and the migration distance was shortened ([147.10±53.74] μm vs. [98.03± 40.95] μm, $P<0.0001$), with all the differences being statistically significant. Compared with those cultured with the low-stiffness type Ⅰ collagen gel, NK-92MI cells cultured with high-stiffness type Ⅰ collagen gel for 24 h could promote DLD-1 cell proliferation, with the proliferation rate being (46.39±12.79)% vs. (65.87±4.45)% ($P<0.05$) and reduce the cell killing ability. Comparison of the cells cultured for 48 h led to similar results, with the proliferation rates

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being (31.36±2.88)% vs. (74.57±2.16)% (P<0.05), and the differences were all statistically significant. **Conclusion** The 3D culture environment of type Ⅰ collagen gels with different levels of stiffness alters the morphology, migration ability, and killing function of NK-92MI cells. This study provides the research basis for exploring and understanding the mechanisms by which the biomechanical microenvironment affects the immune response of NK cells, as well as laying the theoretical foundation for optimizing immunotherapy protocols.

【Key words】 Collagen type Ⅰ Extracellular matrix Mechanics Killer cells Immune response

在机体免疫应答发生过程中,免疫细胞会面临复杂的生化和物理环境。力学免疫学研究发现,生化信号已经无法完全解释细胞复杂的生物学行为。感知和响应邻近细胞及细胞外基质(extracellular matrix, ECM)的力学信号对免疫细胞的功能至关重要,包括刚度、拓扑结构、黏弹性等[1]。在力学信号转导过程中,细胞可以将外部生物物理刺激转化为细胞内生化信号,从而引起细胞骨架重组、基因调控和/或染色质的表观遗传修饰[2]。

自然杀伤(natural killer, NK)细胞是人体内非常重要的免疫细胞,其无需对抗原预先识别即可高效、非特异性的杀伤靶细胞,是先天性免疫系统的核心成员。在生理或病理组织中NK细胞可受到不同的力学刺激,如血液的流体剪切力,组织的固体应力等,这些力学信号可调控NK细胞免疫活性。研究发现,随着肿瘤基质和靶细胞的力学特性改变,NK细胞的浸润会发生显著的变化[3]。流体剪切力可通过NKG2D介导的力学感应增强NK细胞对循环肿瘤细胞的杀伤作用[4]。此外,NK细胞在不同刚度基底上表现出刚度依赖性激活[5]。以上研究表明,力学刺激对NK细胞免疫功能的调节具有重要作用。

目前,许多研究仍基于二维(two-dimensional, 2D)培养模型观察力学因素对细胞的迁移、增殖、分化等过程的影响。然而,机体内细胞处于复杂的三维(three-dimensional, 3D)微环境。与传统2D培养相比,3D培养中细胞的力学微环境更接近体内环境[6]。3D微环境基质刚度对NK细胞的迁移及细胞杀伤作用尚不清楚。因此,本研究使用大鼠尾Ⅰ型胶原蛋白构建不同刚度的胶原凝胶,探索3D培养微环境基质刚度对NK-92MI细胞的形态、自由迁移和细胞杀伤能力的影响,为更好的开发基于NK细胞的免疫治疗奠定基础。

1 材料与方法

1.1 材料

1.1.1 细胞和实验动物

NK-92MI细胞购自美国ATCC,人结直肠腺癌上皮细胞DLD-1由贵州医科大学赵星教授课题组所赠。SPF级Sprague Dawley(SD)大鼠,雌雄不拘,购自辽宁长生生物技术股份有限公司,许可证号:SCXK(辽)2015-0001。

1.1.2 主要试剂与仪器

α-MEM培养基、胎牛血清(Gibco,美国),RPMI1640培养基(Hyclone,美国),马血清、胶原酶(北京索莱宝,中国);CCK-8试剂盒(同仁化学,中国);叶酸、肌醇和β-巯基乙醇(Sigma,美国);高内涵成像系统(Image Xpress Micro 4,美国);激光共聚焦显微镜(Nikon-AX R,日本);流变仪(Anton Paar,奥地利);二氧化碳恒温培养箱(Thermo Fisher,美国);酶标仪(bio-rad,美国)。

1.2 方法

1.2.1 细胞培养

用含2 mmol/L谷氨酰胺、0.2 mmol/L肌醇、0.02 mmol/L叶酸、0.1 mmol/L β-巯基乙醇、12.5%胎牛血清、12.5%马血清,以及1%青霉素-链霉素的α-MEM培养基将NK-92MI细胞培养于37 ℃、体积分数5%CO_2的培养箱。待细胞融合至90%后制成细胞悬液接种于Ⅰ型胶原凝胶中。用含10%胎牛血清、1%青霉素-链霉素的RPMI1640培养基将DLD-1细胞培养于37 ℃、体积分数5%CO_2的培养箱。

1.2.2 鼠尾Ⅰ型胶原蛋白提取及胶原凝胶力学表征

在文献方法基础上进行优化提取鼠尾胶原蛋白[7]。选取健康SD大鼠麻醉后处死,从尾肌腱中分离出Ⅰ型胶原,用0.1%无菌乙酸溶解后,15 000 r/min离心1 h,取上清,透析8次后冻干。取适量冻干样品,用0.1%无菌乙酸溶解,制备成10 mg/mL的胶原蛋白溶液,将胶原蛋白溶液与适量10×α-MEM及细胞培养液混合分别制备1 mg/mL(低刚度)和4 mg/mL(高刚度)的预混合溶液,用0.1 mol/L NaOH调节预混液pH值为7.4, 37 ℃固化1 h,获得低刚度和高刚度Ⅰ型胶原凝胶。在倒置激光共聚焦显微镜下用激发光561 nm成像,记录样品连续横截面图像,观察Ⅰ型胶原凝胶的纤维结构。所有凝胶的图像采集参数均保持一致。

将低刚度和高刚度Ⅰ型胶原凝胶分别进行振幅扫描和频率扫描测量刚度。选用PP25不锈钢圆板(1 mm直径),测试间隙设置为1 mm,振幅扫描测试程序设定振幅应变范围为0.1% ~ 200%,角频率为10 rad/s,测试温度为25 ℃,法向应力FN为0 N。频率扫描测试程序设定角频率为0.1 ~ 100 rad/s,应变保持1%,测试温度为25 ℃,法向

应力FN为0 N。储能模量(G')反映凝胶的弹性特征,损耗模量(G")反映该凝胶的粘性特征,取测试结果中处于平台期的储能模量(G')均值,即为该凝胶的刚度值。

1.2.3　NK-92MI细胞形态及迁移分析

低刚度Ⅰ型胶原凝胶:取100 μL Ⅰ型胶原溶液(10 mg/mL)与10 μL 10×α-MEM混合,再加入约90 μL 0.1 mol/L NaOH,搅拌均匀,使预混液pH值变为7.4,随后将800 μL NK-92MI细胞悬液(约1×10^6细胞)与上述预混液充分混合。

高刚度Ⅰ型胶原凝胶:取400 μL Ⅰ型胶原溶液(10 mg/mL)与40 μL 10×α-MEM混合,再加入约160 μL 0.1 mol/L NaOH,搅拌均匀,使预混液pH值变为7.4,随后将400 μL NK-92MI细胞悬液(约1×10^6细胞)与上述预混液充分混合。

分别取100 μL上述两组预混液接种于96孔板,37 ℃孵育1 h,待胶原溶液聚合成凝胶后补充完全培养基,转移至37 ℃,体积分数5%CO_2的培养箱中培养24 h。将96孔板置于倒置显微镜下采集细胞图像,Image J (v2.1.0,美国)软件对图像进行处理,分析细胞面积、圆度和长短轴比。

NK-92MI细胞的迁移能力是其在体内高效巡逻、发挥免疫监视功能的基础。本研究采用高内涵成像系统分析不同刚度Ⅰ型胶原凝胶对NK-92MI细胞迁移能力的影响。将96孔板放入Image Xpress Micro 4高内涵显微镜采集NK-92MI细胞迁移图像,时间间隔为1 s,连续采集1 h。使用Image J中Manual Tracking plugin跟踪NK-92MI细胞的迁移,chemotaxis and migration tool (IBIDI公司)构建细胞迁移轨迹、分析迁移速度及迁移距离,DiPer软件分析均方位移(mean square displacement, MSD)[8]。

1.2.4　NK-92MI细胞杀伤能力检测

为了评估Ⅰ型胶原凝胶刚度对NK-92MI细胞杀伤活性的影响,本研究收集不同刚度Ⅰ型胶原凝胶中培养24、48 h的NK-92MI细胞,并分别与DLD-1细胞共培养,利用CCK8法检测DLD-1细胞的增殖情况。将NK-92MI细胞分别在低刚度和高刚度Ⅰ型胶原凝胶中培养24、48 h后,用1%的胶原酶溶解胶原,获得NK-92MI细胞悬液,PBS洗涤后,将获得的NK-92MI细胞与DLD-1以5∶1数量接种于U形底96孔板(靶效细胞孔),每孔100 μL,同时另外分别接种相同数量NK-92MI细胞(效应细胞孔)和DLD-1细胞(正常靶细胞孔)于96孔板,于37 ℃,体积分数5%CO_2共培养4 h后,每孔加入20 μL CCK-8试剂,37 ℃孵育2 h,在酶标仪450 nm处测定OD值。DLD-1细胞增殖率(%)=(靶效细胞孔OD值−效应细胞孔OD值)/正常靶细胞孔OD值。

1.2.5　统计学方法

定量数据以$\bar{x}\pm s$表示。所有实验数据统计分析均采用SPSS软件(v27.0.1,美国IBM)进行,两组间比较采用Dunnett-t检验,多组比较采用单因素方差分析(ANOVA),$P<0.05$为差异有统计学意义。

2　结果

2.1　Ⅰ型胶原凝胶制备及表征

结果显示,低刚度Ⅰ型胶原凝胶(1 mg/mL)和高刚度Ⅰ型胶原凝胶(4 mg/mL)均可以形成稳定的3D凝胶网络结构(图1A-1B)。流变仪测定Ⅰ型胶原凝胶的力学性能结果显示,凝胶储能模量(G')大于损耗模量(G"),提示凝胶主要发生弹性形变,呈现固态特征;高刚度Ⅰ型胶原凝胶平台期的储能模量(G')为(114.50±3.40) Pa,低刚度Ⅰ型胶原凝胶平台期的储能模量(G')为(10.97±2.10) Pa,差异有统计学意义($P<0.0001$,图1C-1F)。

2.2　Ⅰ型胶原凝胶刚度影响NK-92MI细胞的形态

倒置显微镜下,与低刚度Ⅰ型胶原凝胶相比较,高刚度Ⅰ型胶原凝胶中NK-92MI细胞形态更加细长(图2A);低刚度Ⅰ型胶原凝胶中NK-92MI细胞的平均面积相为(69.88±26.97) μm^2,而高刚度Ⅰ型胶原凝胶中NK-92MI细胞的平均面积减少为(46.59±21.62) μm^2,差异有统计学意义($P<0.05$);与低刚度Ⅰ型胶原凝胶相比较,高刚度Ⅰ型胶原凝胶中NK-92MI细胞的细胞圆度明显减小(0.82±0.12 vs. 0.78±0.18, $P<0.05$),而高刚度Ⅰ型胶原凝胶中细胞的长短轴比(1.82±0.72)较低刚度Ⅰ型胶原凝胶中(1.51±0.39)增加($P<0.0001$,图2B-2D)。

2.3　Ⅰ型胶原凝胶刚度影响NK-92MI细胞的迁移能力

与低刚度Ⅰ型胶原凝胶相比较,高刚度Ⅰ型胶原凝胶可显著抑制NK-92MI细胞的迁移(图3)。图3A为显微镜记录下的细胞运动轨迹,图3B为将细胞运动轨迹转换为从原点发出的轨迹鸟瞰图,可直观观察NK-92MI细胞的迁移情况。NK-92MI细胞在高刚度Ⅰ型胶原凝胶中的自由迁移速度为(1.70±0.72) μm/min,迁移距离为(98.03±40.95) μm。而其在低刚度Ⅰ型胶原凝胶中的迁移速度为(2.50±0.91) μm/min,迁移距离为(147.10±53.74) μm ($P<0.001$,图3C-3D)。在高刚度的Ⅰ型胶原凝胶中NK-92MI细胞的均方位移小于低刚度胶原基质中的细胞(图3E)。

2.4　Ⅰ型胶原凝胶刚度调节NK-92MI细胞的杀伤活性

CCK8法检测结果发现,经高刚度Ⅰ型胶原凝胶培养

图 1 不同刚度Ⅰ型胶原凝胶网络形态及储能模量

Fig 1 Characterization of the morphology and rheological properties of 3D collagen gel networks of varying stiffness

A, Images of the structure of collagen gel fibers in monolayers of different stiffness obtained using laser scanning confocal microscopy with reflected light. The images show the arrangement of fibers within collagen gels of different stiffness. B, 3D reconstructions of collagen gels of different stiffness. C, Dynamic strain scans of collagen gels of different stiffness. D, Dynamic frequency scans of collagen gels of different stiffness. E-F, Statistical plots of C and D. L: low-stiffness group; H: high-stiffness group, n=3. **** P<0.0001.

24 h、48 h的NK-92MI细胞与DLD-1细胞连续共培养后，DLD-1细胞增殖率分别为(65.87±4.45)%和(74.57±2.16)%，均高于经低刚度Ⅰ型胶原凝胶培养24 h、48 h的NK-92MI细胞共培养的DLD-1的增殖率〔(46.39±12.79)%、(31.36±2.88)%〕，差异有统计学意义(P< 0.05，图4)。

3 讨论

尽管NK细胞治疗作为有巨大潜力的癌症免疫疗法之一，并在基础及临床研究中取得了一系列重要进展，但在面对实体肿瘤时仍有许多难题亟待解决，如实体肿瘤中NK细胞浸润数量少，NK细胞杀伤活性低等[9]。近年来，人们逐渐将焦点从化学因素转向细胞外的力学因素对NK细胞的影响。研究表明，在生理或病理状态下机体的力学特性会发生显著的改变，如器官纤维化或实体肿瘤微环境中Ⅰ型胶原的异常交联、沉积导致其弹性模量显著增加，从而引起ECM整体刚度增高[10]。本研究通过构建不同刚度Ⅰ型胶原凝胶，探索其对NK-92MI细胞的形态、自由迁移能力和细胞杀伤作用的影响。

研究结果显示，随着Ⅰ型胶原凝胶刚度增加，NK-92MI细胞的圆度指数及细胞面积降低，部分细胞由圆形变成长梭形。细胞长短轴比也随着Ⅰ型胶原凝胶刚度增加而增加，表明基质刚度对细胞形态具有调节作用。细胞的形态主要受骨架蛋白调控，细胞骨架可以感知和适应外部的力学信号。研究发现，不同的基质刚度可引起细胞肌动蛋白骨架发生双相流变，从软底物上的流体转变为硬底物上的固体，软基质上生长的细胞与细胞外基质的低摩擦和肌动蛋白径向流动有利于细胞维持圆形形状[11]。肌动蛋白是细胞骨架的主要成分之一，NK细胞在迁移以及与靶细胞结合过程中，通过聚合和解聚，从球状肌动蛋白变为纤维状肌动蛋白(fibrous actin, F-actin)，而F-actin解聚在NK细胞发挥细胞毒作用中具有重要作用[12]。由此可见，细胞与ECM的锚定涉及细胞形态的变化，这些变化可能会在细胞内产生相应的机械应力，从而影响细胞的生物学功能。

免疫系统的正常运转依赖于免疫细胞在机体内快速、高效、协调地迁移。因此，在本研究中检测不同刚度Ⅰ型胶原凝胶对NK-92MI细胞迁移的影响。结果显示，随着胶原基质刚度的增加，NK-92MI细胞的迁移速度及累计迁移距离均显著减弱。类似地，巨噬细胞的迁移能力也随着三维基质刚度的增加而显著降低[13]。此外，对小鼠癌症模型的研究表明，ECM可以影响肿瘤内T细胞的迁移[14]。有研究发现，增加NK细胞的速度和持久性可

图 2　Ⅰ型胶原凝胶刚度对NK-92MI细胞形态的影响

Fig 2　Effect of 3D collagen gel stiffness on NK-92MI cell morphology

A, Images were captured after culturing NK-92MI cells in 3D collagen gels of different levels of stiffness for 24 hours (scale bar=20 μm). Cell morphological parameters were analyzed using ImageJ software. B, Cell area. C, Cell circularity (circularity ranges from 0 to 1, where 1=perfect circle). D, Cell aspect ratio. L: low-stiffness group; H: high-stiffness group, n=3. * P<0.05, **** P<0.000 1.

图 3　Ⅰ型胶原凝胶刚度刚度对NK-92MI细胞迁移能力的影响

Fig 3　Effect of 3D collagen gel stiffness on the migration ability of NK cells

A, Bright field static image of the last frame of the time-lapse video of NK cell migration (scale bar=40 μm). B, Convertion of the trajectories in Figure A to an aerial view of all trajectories emanating from the origin. C, Cell migration distance. D, Cell migration rate. E, Mean azimuth shift of cell migration. L: low-stiffness group; H: high-stiffness group, n=3. *** P<0.001, **** P<0.000 1.

图 4　不同刚度Ⅰ型胶原凝胶中NK-92MI细胞对DLD-1细胞增殖的影响

Fig 4　Effect of NK-92MI cells in collagen gels with different stiffness on the proliferation of DLD-1 cells

L: low-stiffness group; H: high-stiffness group, n=3. * P<0.05, *** P<0.001.

以减少它们搜索目标细胞的时间，从而提高NK细胞的杀伤效率[15]。因此，笔者认为较高的基质刚度可抑制细胞扩散的动力学，降低NK细胞的杀伤效率。

尽管NK细胞对肿瘤细胞具有较强的细胞毒能力，但肿瘤细胞和肿瘤微环境可以通过多种机制来诱导NK细胞的功能障碍，从而肿瘤细胞免受NK细胞的攻击[16]。本研究发现，随着Ⅰ型胶原凝胶刚度的增加，NK-92MI细胞的细胞杀伤能力显著降低。力学环境的变化可以通过多种机制调节免疫应答，ECM过度重塑会影响肿瘤组织中T细胞的活性，从而影响免疫检查点抑制剂的反应[17]。除此之外，基质刚度的变化可以调控巨噬细胞的分化方

向,从而调节免疫应答[18]。本课题组前期研究发现3D水凝胶能够通过力学敏感信号分子RhoA和CDC42显著改变树突状细胞(dendritic cells, DCs)的力学表型,从而调控DC的免疫应答[19-20]。通常情况下,NK细胞杀伤靶细胞的基础是形成稳定的免疫突触,其中涉及黏附分子和细胞骨架重塑介导的免疫黏附等力学事件[21]。由于黏附分子、细胞骨架相关调控蛋白是细胞响应力学信号过程中的力学敏感分子,因此,3D培养环境刚度对NK细胞的骨架蛋白及黏附分子的调节可能是减弱NK细胞杀伤作用的主要原因。

综上所述,Ⅰ型胶原凝胶3D培养微环境基质刚度可影响NK-92MI细胞的形态、降低NK-92MI细胞迁移能力和细胞杀伤能力。该研究从力学免疫学的角度出发,拓展了研究者对基质刚度影响NK细胞生物学行为的了解,为进一步研究NK细胞力-化耦合分子机制,开发基于NK细胞更好的免疫治疗策略奠定基础。

* * *

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